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NEW INSIGHTS ON EMERGENCE AND SUCCESS OF CARBAPENEM-RESISTANT *ACINETOBACTER* SPP.

Tese do 3º Ciclo de Estudos Conducente ao Grau de Doutoramento em Segurança e Saúde Ocupacional na especialidade de Microbiologia

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Aos meus pais, irmãos e sobrinhos,
Aos meus amigos de sempre.

"He who see things grow from the beginning will have the best view of them..."
Aristóteles

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"Sou o resultado da confiança e da força de cada um de vocês"
(Augusto Branco)

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ABSTRACT

The rise on antimicrobial resistance rates among main bacterial pathogens is a growing challenge for healthcare institutions all over the world. *Acinetobacter,* and particularly *Acinetobacter baumannii*, are among the most concerning bacteria, being associated with a prodigious ability to persist in the hospital environment and to become resistant to multiple antimicrobial agents, including the most relevant and last-resource options, such as carbapenems.

Although *A. baumannii* is still considered the most prevalent *Acinetobacter* species within the hospital setting, the reports of infections caused by non-*baumannii* species, including multidrug resistant strains, increased in the last years. In Portugal, besides the endemicity of carbapenem-hydrolyzing class D β-lactamase (CHDL)-producing *A. baumannii* isolates observed for more than two decades, this scenario seems to be worsening, with the increase of isolates with an enlarged antibiotic resistance profile, being needed further clarification regarding their clonality and antimicrobial resistance mechanisms. Moreover, other clonal features (e.g. virulence or resilience traits), as well as relevance of other settings for the emergence and proliferation of particular *A. baumannii* clones, are still poorly understood. Additionally, the interspecies transfer of resistance traits that occur between non-*baumannii* and *A. baumannii* species also account for carbapenem resistance, with non-*baumannii* species/non-hospital niches potentially constituting an important source/reservoir of relevant resistance genes that worth to be explored.

The increasing availability of whole genome sequencing (WGS) provided an opportunity for defining these organisms' evolutionary processes, and also for the recognition of dispersion drivers/transmission pathways, associated with antibiotic resistance genes. However, most of these methods are still expensive and time-consuming which is incompatible with a quick infection control surveillance (mainly in an outbreak context). Thus, alternative high-throughput quick and low-cost methods are highly demanded.

The **main goals** of this work were to identify the features contributing for the clonal dynamics and success of particular *A. baumannii* lineages that were observed in Portuguese clinical settings since 1995; to understand the role of *non-baumannii Acinetobacter* species in the carbapenem resistance dissemination; to unveil unrecognized niches for *Acinetobacter*; and to explore the potential of high-throughput omic technologies [WGS, Fourier transform infrared (FTIR) spectroscopy, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF MS)], for an accurate and quick typing of *Acinetobacter*. Moreover, combining information of these different approaches to identify bacterial cell components explain the discriminatory ability of MALDI-TOF MS and FTIR spectroscopy. To accomplish these purposes, a multilevel characterization (including bacterial species, clones, and genomic pieces contributing to virulence and adaptation to different stressors), of a recent carbapenem-resistant *Acinetobacter* spp. Portuguese clinical collection (hospital, LTCF and community laboratory; 2010-2015), was performed. Additionally, for a more comprehensive analysis, whole genome sequences

of representatives of this collection and of older Portuguese isolates were compared. Moreover, the occurrence, diversity and antimicrobial resistance of *Acinetobacter* spp. in non-clinical clinical niches of an African country was also evaluated. Finally, a representative national and international collection of *Acinetobacter* species from the ACB complex was used to assess the potential of FTIR-ATR and MALDI-TOF MS for these species identification and typing.

The **results** of this Thesis revealed that, since 2010, ST218 gradually replaced the previously dominant ST208, being now the main clone associated with carbapenem-resistance among Portuguese clinical settings. The extended resistance profile of ST218 isolates affecting all aminoglycosides (possibly associated with the presence of *armA*, *aph(3´)-Ia and aac(6´)-Ib* genes), together with an enlarged virulence potential (includes heme oxygenase) and a capsular type (displaying legionaminic acid) more adapted to the host colonization, were found to be the main advantages of this clone. Interestingly, the chromosome located $bla_{\text{OXA-23}}$, associated with Tn*2006* is still the most prevalent carbapenem resistance determinant associated with *A. baumannii*. Moreover, a clear potential of non-*baumannii* species to acquire and act as a source/reservoir of carbapenem-resistance determinants, with potential for further dissemination was evidenced, being the description of new CHDL-carrying plasmids among *A. pittii* clinical isolates (including a conjugative $bla_{\text{OXA-23}}$-plasmid) and the detection of *A. bereziniae* carrying $bla_{\text{IMP-5}}$ plasmid-encoded, important findings. The observation of CHDL-*Acinetobacter* spp. producers in non-clinical niches in an African country indicates the ongoing selective pressure by unrecognized agents. Moreover, in this region, a possible water–human transmission cycle for *Acinetobacter* spp., including CHDL producers, was observed. Finally, it was demonstrated the potential of FTIR for the identification of ACB closely related species, and also for the discrimination of clinically relevant *A. baumannii* clones. Conversely, MALDI-TOF MS showed a good suitability for ACB species identification but a limited potential to discriminate MDR *A. baumannii* clones, apparently explained by the high degree of conservation of ribosomal proteins.

This Thesis identified important features contributing for the emergence and success of carbapenem-resistant *Acinetobacter* spp. in the clinical setting, the non-*A. baumannii* species contribution for the dissemination of important carbapenemases among the genus *Acinetobacter*, and unveiled a possible significant human exposition through water consumption to these worrisome resistant bacteria in an African country. Moreover, the suitability of FTIR spectroscopy and MALDI-TOF MS for species and clonal discrimination with potential application for routine microbiology laboratories is demonstrated.

RESUMO

O aumento da resistência a antimicrobianos entre as principais bactérias patogénicas é um desafio cada vez maior para os serviços de saúde em todo o mundo. *Acinetobacter*, e em particular *Acinetobacter baumannii*, é considerado um grupo preocupante de bactérias, dada a sua extraordinária capacidade em persistir no ambiente hospitalar e adquirir resistência a múltiplos agentes antimicrobianos, incluindo as opções de última linha, como é o caso dos carbapenemos.

Embora *A. baumannii* ainda seja a espécie mais frequente em ambiente hospitalar, nos últimos anos assistiu-se a um aumento de infeções causadas por outras espécies de *Acinetobacter*, incluindo isolados resistentes a múltiplos antibióticos. Em Portugal, para além da endemicidade de clones de *A. baumannii* produtores de carbapenemases de classe D (CHDL), observada há mais de duas décadas, verifica-se atualmente um agravamento deste cenário em resultado do aparecimento de isolados resistentes a um maior número de antibióticos, sendo necessário elucidar quais os clones e mecanismos de resistência associados a este comportamento. Adicionalmente, características de virulência e resiliência, bem como a contribuição de nichos não hospitalares no aparecimento e disseminação de clones particulares de *A. baumannii*, foram ainda pouco exploradas. Acresce ainda que, a contribuição de espécies não-*baumannii* e nichos não hospitalares como fontes/reservatórios de genes de resistência relevantes carece de elucidação.

A crescente utilização da sequenciação do genoma completo (WGS) tem proporcionado a definição de processos evolutivos em diferentes organismos, assim como o reconhecimento de mecanismos e vias de transmissão e disseminação da resistência a antibióticos. No entanto, tratam-se de métodos caros e demorados, o que é incompatível com uma vigilância rápida, essencial para o controlo de infeção (principalmente em situações de surto). Por isso, torna-se imprescindível o desenvolvimento de métodos alternativos de alto rendimento, que sejam rápidos, precisos e de baixo custo.

Este trabalho teve como **principais objetivos** a identificação das características que contribuem para a dinâmica clonal e o sucesso das linhagens de *A. baumannii* detetadas, desde 1995, nos hospitais Portugueses; compreender o papel das espécies de *Acinetobacter* não-*baumannii* na disseminação da resistência aos carbapenemos; reconhecer novos nichos para *Acinetobacter* e explorar o potencial de tecnologias de alto rendimento [WGS, Espectroscopia de Infravermelho com Transformada de Fourier (FTIR), "Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry" (MALDI-TOF MS], para uma caracterização mais rápida e precisa de *Acinetobacte*r, contribuindo para o reconhecimento das características bacterianas subjacentes à discriminação obtida com essas metodologias.

Para o efeito, foi efetuada numa coleção recente de isolados clínicos (hospital, LTCF e laboratório comunitário, 2010-2015) de *Acinetobacter* spp. resistentes aos carbapenemos, uma caracterização a múltiplos níveis (espécies, clones e elementos genéticos putativamente envolvidos na virulência e adaptação a diferentes agentes seletivos). Adicionalmente, para uma análise mais abrangente, dados de WGS de isolados representativos desta coleção e de isolados Portugueses mais antigos foram comparados. Além disso, a ocorrência, diversidade e resistência a

antimicrobianos de uma coleção de isolados não clínicos de *Acinetobacter* spp. de um país africano foram também avaliadas. Por fim, uma coleção representativa de isolados nacionais e internacionais pertencentes a espécies de *Acinetobacter* do complexo *Acinetobacter calcoaceticus-baumannii* (ACB) foi utilizada para avaliar o potencial de FTIR-ATR e MALDI-TOF MS na discriminação dessas espécies.

Os **resultados** obtidos no âmbito desta Tese revelaram que, desde 2010, o **ST218** substituiu gradualmente o ST208 anteriormente dominante, sendo agora o principal clone associado à resistência ao carbapenemos em diferentes contextos clínicos portugueses. Comparando os dois clones, verificou-se que o ST218 apresentava vantagens relativamente ao clone anterior, nomeadamente um perfil de resistência aumentado afetando todos os aminoglicosídeos (possivelmente associados à presença dos genes *armA*, *aph(3´)-Ia* e *aac(6´)-Ib*), um maior potencial de virulência (incluindo a presença de heme oxigenase) e um tipo capsular (apresentando o ácido legionamínico) mais adaptado à colonização do hospedeiro. Curiosamente, o gene $bla_{\text{OXA-23}}$ cromossómico, associado ao Tn*2006* continua a ser o determinante de resistência aos carbapenemos mais frequente em *A. baumannii*. Adicionalmente, verificou-se um claro potencial das espécies não-*baumannii* para adquirir e/ou atuar como fonte/reservatório de determinantes de resistência aos carbapenemos, o que pode resultar num considerável aumento da sua disseminação. São exemplos a descrição de novos plasmídeos associados a CHDL em isolados clínicos de *A. pittii* (incluindo um plasmídeo conjugativo com $bla_{\text{OXA-23}}$) e a deteção de *A. bereziniae* resistentes aos carbapenemos em resultado da presença de $bla_{\text{IMP-5}}$ plasmídica. A deteção de isolados de *Acinetobacter* spp. produtores de CHDL em isolados clínicos de África indicia uma contínua pressão seletiva por agentes ainda não determinados. Para além disso, parece ocorrer nesta região um ciclo de transmissão água-humanos de *Acinetobacter* spp., incluindo isolados produtores de CHDL. Finalmente, foi demonstrado o potencial do FTIR para a identificação de espécies estreitamente relacionadas pertencentes ao complexo ACB e também para a discriminação de clones de *A. baumannii* clinicamente relevantes. Por outro lado, o MALDI-TOF MS mostrou uma boa adequação para a identificação de espécies de ACB, mas um potencial limitado para discriminar clones de *A. baumannii*, provavelmente devido ao alto grau de conservação das proteínas ribossómicas nessas espécies.

Esta Tese identificou características importantes que contribuem para a emergência e sucesso de *Acinetobacter* spp. resistentes aos carbapenemos em contexto hospitalar, a contribuição de espécies não-*baumannii* para a disseminação de carbapenemases importantes no género *Acinetobacter*, revelando uma possível e potencialmente grave exposição humana, em África, a *Acinetobacter* spp. resistentes através do consumo de água. Para além disso, foi demonstrada a adequação da espectroscopia de FTIR e MALDI-TOF MS para a identificação de espécies e discriminação clonal em *Acinetobacter* com potencial aplicação em laboratórios de rotina.

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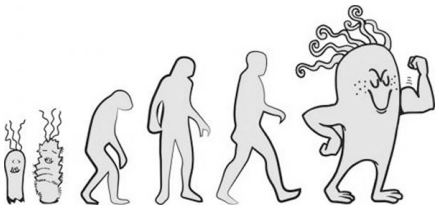
	Description
3-LST	3-locus sequence typing
AAC	Aminoglycoside acetyltransferases
AAD	Aminoglycoside adenyltransferase
AbaR	*A. baumanni* resistance island
ABC	ATP-binding cassette transporter
AbeM	*A. baumannii* efflux pump of MATE family
AbeS	*A. baumannii* efflux pump of SMR family
ACB	*Acinetobacter calcoaceticus-Acinetobacter baumannii*
AceI	*Acinetobacter* chlorhexidine efflux protein
Ade	*A. baumannii* multidrug-resistant efflux pump
AFLP	Amplified Fragment Length Polymorphism
AME	Aminoglycoside-modifying enzymes
ANT	Aminoglycoside adenyltransferases
APH	Aminoglycoside phosphotransferases
ARDRA	Amplified 16S ribosomal DNA restriction analysis
ATR	Attenuated total reflectance
BIGSdb	Bacterial Isolate Genome Sequence Database
BSAC	British Society for Antimicrobial Chemotherapy
CarO	Carbapenem-associated outer membrane protein
CC(s)	Clonal Complex(es)
cgMLST	Core genome Multilocus sequence typing
CHDL(s)	Carbapenem-hydrolysing class D-β-lactamase(s)
CLSI	Clinical and Laboratory Standards Institute
CmlA	Chloramphenicol resistance *Acinetobacter*
CPS	Capsular polysaccharide
CraA	Chloramphenicol resistance *Acinetobacter* pump
CTn(s)	Conjugative transposon(s)
DDH	DNA-DNA hybridization
DNA	Deoxyribonucleic acid
EC(s)	European clone(s)
ECDC	European Center for Disease Prevention and Control
ECOFF(s)	Epidemiological Cut-Off(s)
EDTA	Ethylenediaminetetraacetic acid
ESBL(s)	Extended-Spectrum β-Lactamase(s)
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FolA	Folate
FTIR	Fourier transform infrared
GI(s)	Genomic island(s)
GyrA/ParC	DNA Gyrase/partitioning of the nucleoid partition
HGT	Horizontal gene transfer
IC(s)	International clone(s)
ICUs	Intensive care units
Inc	Incompatibility
IS(s)	Insertion Sequence(s)
KL-type	Capsular types
LPS	Lipopolysaccharide
LTCF(s)	Long-Term Care Facility(ies)
MacB	Macrolide export ATP-binding/permease protein
MALDI-TOF MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry

Description	
MATE	Multidrug and toxic compound extrusion efflux pump
MBL(s)	Metallo-β-lactamase(s)
MDR	Multidrug resistant
MFS	Major facilitator superfamily
MGE(s)	Mobile Genetic Element(s)
MIC	Minimum Inhibitory Concentration
MLSA	Multilocus sequence analysis
MLST	Multilocus sequence typing
MLVA	Multiple-locus variable-number tandem repeat analysis
MOB	Plasmid mobility genes
MPF	Mating pair formation
NCBI	National Center for Biotechnology Information
Omp	Outer membrane protein
OMV(s)	Outer membrane vesicle(s)
OXA(s)	Oxacillinase(s)
PBPs	Penicillin Binding Proteins
PBRT	Plasmid-based replicon typing scheme
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
PCR-ESI/MS	Multilocus PCR/electrospray ionization mass spectrometry
PFGE	Pulsed-Field Gel Electrophoresis
PLSDA	Partial least squares discriminant analysis
RAPD	Random Amplification of Polymorphic DNA
Rep	Replicase
RepA	Enzyme required for homologous recombination and recombination repair
rep-PCR	Repetitive element palindromic PCR
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RND	Resistance-nodulation-cell division
rRNA	Ribosomal ribonucleic acid
SBT	Sequence-based typing
SMR	Small multidrug-resistant efflux pump
SNP(s)	Single nucleotide polymorphism(s)
ST(s)	Sequence Type(s)
T4CP	Type IV coupling proteins
T4SS	Type 4 secretion system
Tn(s)	Transposon(s)
VNTR	Variable-number tandem repeat
WGS	Whole-genome sequencing
WHO	World Health Organization
XDR	Extensively drug-resistant



Chapter 1

Introduction

"The best way to get something done is to begin."

Anonymous author

1.1. The genus *Acinetobacter*

1.1.1. The challenging taxonomy of *Acinetobacter*

"Taxonomy is described sometimes as a science and sometimes as an art, but really it´s a battleground."

Bill Bryson

The history of *Acinetobacter* genus is very ancient, being predicted that its last common ancestor was a close contemporary of the last common ancestor of *Enterobacteriaceae,* with this antiquity explaining *Acinetobacter* genus metabolic and ecological diversity [1]. However, an accurate dating is almost impossible given the lack of fossil records [1]. The first description of an *Acinetobacter* organism have occurred in 1911, when the botanist Martinus Beijerinck isolated *Micrococcus calcoaceticus* from the soil [2]. In 1954, Brisou and Prevot proposed the current genus designation, *Acinetobacter* (from the Greek words "*akinetos*" and "*bactrum*", which means non-motile and rod, respectively), composed by non-pigmented Gram-negative bacteria and comprising both oxidase-negative and oxidase-positive species [3]. A few years later, in 1971, based on several studies performed by Paul Baumann, the Subcommittee on the taxonomy of *Moraxella* recommended that the genus *Acinetobacter* should include only oxidase-negative strains [4,5]. Currently, *Acinetobacter* genus is included in the Kingdom *Bacteria*, Phylum *Proteobacteria*, Class *Gammaproteobacteria*, Order *Pseudomonadales* and Family *Moraxellaceae*, comprised by Gram-negative non-fermenting coccobacilli, with a DNA G+C content of 39-47%, strictly aerobic, catalase positive and oxidase negative [6]. More than 60 named and unnamed species (including strains not yet recognized by the International Committee on Systematic Bacteriology, Table 1) were described among this genus, in different environments (e.g. as inhabitants of human and other animal guts, in sewage, water, soil, food and also as important agents of opportunistic infections in humans) (Table 1). The taxonomy of *Acinetobacter* still suffers from unclear taxonomic positions, confusing nomenclature of some provisional species, high number of unidentifiable environmental strains and a number of controversial interpretations of taxonomic data [7,8]. Thus, understanding the diversification of this genus is, currently, a real challenge.

The following sections, include an update overview of the main methodologies used for *Acinetobacter* identification (at species level) along with the evolution and

applications of the typing methods used for the characterization of *Acinetobacter* populations (mainly among *A. baumannii*).

TABLE 1. Species currently described within *Acinetobacter* genus

Species	Genomic species[a]	Origin of isolation	Type Strain	References
Acinetobacter albensis		Soil, water	ANC 4874^T; CCUG 67281^T; CCM 8611^T	[9]
Acinetobacter anitratus		Animal		[10]
Acinetobacter antiviralis*		Tobacco plant	KNF2022^T; KCTC0699BP^T	[11]
Acinetobacter apis*		Intestinal tract of a honey bee	HYN18^T; KACC 16906^T; JCM 18575^T	[12]
Acinetobacter baumannii	2	Mainly human clinical samples	CIP 70.34^T; ATCC 19606^T; DSM 30007	[13]
Acinetobacter baylyi		Activated sludge	B2^T; CIP 107474^T; DSM 14961^T	[14]
Acinetobacter beijerinckii		Human, animal, environmental samples	NIPH 838^T; LUH 4759^T; CCUG 51249^T; CCM 7266^T; 58a^T	[15]
Acinetobacter bereziniae	10	Mainly human clinical samples	LMG 1003^T; CIP 70.12^T; ATCC 17924^T	[16]
Acinetobacter bohemicus		Soil, water	ANC 3994^T; CIP 110496^T; CCUG 63842^T; CCM 8462^T	[17]
Acinetobacter boissieri		Floral nectar of pollinated plants	SAP 284.1^T; LMG 26959^T; CECT 8128^T	[18]
Acinetobacter bouvetii		Activated sludge	4B02^T; DSM 14964^T; CIP 107468^T	[14]
Acinetobacter brisouii*		Peat layer	5YN5-8^T; KACC 11602^T; DSM 18516^T	[19]
Acinetobacter calcoaceticus	1	Soil	ATCC 23055^T; CIP 81.08^T; DSM 30006^T	[20]
Acinetobacter celticus		Soil and water natural ecosystems	ANC 4603^T; CCM 8700^T; CCUG 69239^T; CNCTC 7549^T	[21]
Acinetobacter colistiniresistens	13BJ, 14TU	Human samples	NIPH 2036^T; CCM 8641^T; CIP 110478^T; CCUG 67966^T; CNCTC 7573^T	[22]
Acinetobacter courvalinii	14BJ	Human samples	ANC 3623^T; CCUG 67960^T; CIP 110480^T; CCM 8635^T	[23]
Acinetobacter defluvii*		Hospital sewage	WCHA30^T; CCTCC AB 2016203^T; GDMCC 1.1101^T; KCTC 52503^T	[24]
Acinetobacter dispersus	17	Well water (garden), sewage water, soil (forest), human samples	ANC 4105^T; CCUG 67961^T; CIP 110500^T; CCM 8636^T	[23]
Acinetobacter equi		Horse faeces	114^T; DSM 27228^T; CCUG 65204^T	[25]
Acinetobacter gandensis		Horse dung	UG 60467^T; ANC 4275^T; LMG 27960^T; DSM 28097^T	[26]

TABLE 1 - *Continued*

Species	Genomic species[a]	Origin of isolation	Type Strain	References
Acinetobacter gerneri		Activated sludge	CCM 7198^T; CIP 107470^T; 17A04^T; DSM 14968^T	[14]
Acinetobacter grimontii*[b]/*Acinetobacter junii	5	Activated sludge	17A04^T; DSM 14968^T; CIP 107470^T	[8,14]
***Acinetobacter guangdongensis**[c]*		Lead-zinc ore mine site	1NM-4^T; GIMCC 1.656^T; CCTCC AB 2014199^T; KCTC 42012^T	[27]
Acinetobacter guillouiae	11	Environmental and human samples	LMG 988^T; CIP 63.46^T; ATCC 11171^T; CCUG 2491^T	[16]
Acinetobacter gyllenbergii		Human clinical samples	NIPH 2150^T; RUH 422^T; CCUG 51248^T; CCM 7267^T; 1271^T	[15]
Acinetobacter haemolyticus	4	Sputum	ATCC 17906^T; CIP 64.3^T; DSM 6962^T	[20]
Acinetobacter halotolerans*		Soil	KEMB 9005-333^T; KACC 18453^T; JCM 31009^T	[28]
Acinetobacter harbinensis*		River water	HITLi 7^T; CGMCC 1.12528^T; KCTC 32411^T	[29]
***Acinetobacter indicus**[c]*		Dump site	A648^T; DSM 25388^T; CCM 7832^T	[30]
Acinetobacter johnsonii	7	Mainly human clinical samples	ATCC 17909^T; CIP 64.6^T; DSM 6963^T	[13]
Acinetobacter kyonggiensis*		Sewage treatment plant	KSL5401-037^T; JCM 17071^T; KEMC 5401-037^T	[31]
Acinetobacter kooki		Soil	11-0202^T; KCTC 32033^T; JCM 18512^T	[32]
***Acinetobacter lactucae*/*Acinetobacter dijkshoorniae*[d]**		Iceberg lettuce, human samples	NRRL B-41902^T; CCUG 68785^T; JVAP01^T; CECT 9134^T; LMG 29605^T	[33,34]
Acinetobacter larvae*		Larval gut of *Omphisa fuscidentalis*	BRTC-1^T; ACCC 19936^T; JCM 31367^T	[35]
Acinetobacter lwoffii	8/9	Gangrene	ATCC 9957^T; ATCC 17986^T; CCM 5581^T; CIP 64.10^T; DSM 2403^T	[20]
Acinetobacter marinus*		Sea water	SW-3^T; KCTC 12259^T; DSM 16312^T	[36]
Acinetobacter modestus	18	Sewage water, sludge, human samples	NIPH 236^T; CCUG 67964^T; CIP 110444^T; CCM 8639^T	[23]
Acinetobacter nectaris		Floral nectar	SAP 763.2^T; LMG 26958^T; CECT 8127^T	[18]
Acinetobacter nosocomialis	13TU	Sputum	LMG 10619^T; CCM 7791^T; RUH 2376^T; NIPH 2119^T	[37]
Acinetobacter oleivorans*		Rice paddy	DR1^T; KCTC 23045^T; JCM 16667^T	[38]

TABLE 1 - *Continued*

Species	Genomic species[a]	Origin of isolation	Type Strain	References
Acinetobacter oryzae*		Rice, *Oryza alta*	B23^T; LMG25575^T; CGMCC1.10689^T	[39]
Acinetobacter parvus		Ear of an outpatient	LMG 21765^T; LUH 4616^T; NIPH 384^T; CCM 7030^T	[40]
Acinetobacter pittii	3	Cerebrospinal fluid	LMG 1035^T; CIP 70.29^T; ATCC 19004^T; RUH 2206^T; NIPH 519^T	[37]
Acinetobacter plantarum*		Wheat seedlings plant	THG-SQM11^T; CCTCC AB 2015123^T; KCTC 42611^T	[41]
Acinetobacter populi		*Populus x euramericana* canker	PBJ7^T; CFCC 11170^T; KCTC 42272^T	[42]
Acinetobacter pragensis		Environmental soil and water	ANC 4149^T; CCM 8637^T; CCUG 67962^T; CNCTC 7530^T	[43]
Acinetobacter proteolyticus	19	Human samples	NIPH 809^T; CCUG 67965^T; CIP 110482^T; CCM 8640^T	[23]
Acinetobacter puyangensis		*Populus x euramericana* canker bark	BQ4-1^T; CFCC 10780^T; JCM 18011^T	[44]
Acinetobacter qingfengensis		*Populus x euramericana* canker bark	2BJ1^T; CFCC 10890^T; KCTC 32225^T	[45]
Acinetobacter radioresistens	12	Soil and cotton	FO-1^T; IAM 13186^T	[46]
Acinetobacter rhizosphaerae*		Himalayas desert	BIHB 723^T	[47]
Acinetobacter rudis		Raw milk and raw wastewater	G30^T; LMG 26107^T; CCUG 57889^T; DSM 24031^T; CECT 7818^T	[48]
Acinetobacter schindleri		Urine	LUH 5832^T; NIPH 1034^T; LMG 19576^T; CNCTC 6736^T	[49]
Acinetobacter seifertii	Close to 13TU	Mostly human clinical samples	NIPH 973^T; CIP 110471^T; CCUG 34785^T; CCM8535^T	[50]
Acinetobacter seohaensis*		Sea water	SW 100^T; KCTC 12260^T; DSM 16313^T	[36]
Acinetobacter soli*		Forest soil	B1^T; KCTC 22184^T; JCM 15062^T	[51]
Acinetobacter tandoii		Activated sludge	4N13^T; DSM 14670^T; CIP 107469^T	[14]
Acinetobacter tjernbergiae		Activated sludge	7N16^T; DSM 14971^T; CIP 107465^T	[14]
Acinetobacter towneri		Activated sludge	AB1110^T; DSM 14962^T; CIP 107472^T	[14]
Acinetobacter ursingii/Acinetobacter septicus[e]		Blood	LUH 3792^T; NIPH 137^T; LMG 19575^T; CNCTC 6735^T; AK001^T; DSM 19415^T	[7,49,52]

TABLE 1 - *Continued*

Species	Genomic species[a]	Origin of isolation	Type Strain	References
Acinetobacter variabilis	15	Human and animal samples	NIPH 2171^T; CIP 110486^T; CCUG 26390^T; CCM 8555^T	[53]
Acinetobacter venetianus[f]		Seawater near a beach	RAG-1^T; ATCC 31012^T; CCUG 45561^T; LMG 19082^T; LUH 3904^T; NIPH 1925^T	[54]
Acinetobacter vivianii	20	Sewage water, soil, human samples	NIPH 2168^T; CCUG 67967^T; CIP 110483^T; CCM8642^T	[23]
Unnamed	6	Humans	ATCC 17979^T	[5]
Unnamed	15BJ	Human skin	SEIP 23.78^T	[55]
Unnamed	16BJ	Humans	78	[55]
Unnamed	17BJ	Humans	942	[55]
Unnamed	15TU	Humans, soil, water	M151a^T	[20]
Unnamed	16	Sewage, vegetables, human skin	ATCC 17988^T	[55]
Unnamed	Between 1 and 3	Humans	LUH 1469; 10095^T	[56]

[a] Genomic species according to Bouvet and Grimont classification [13]. BJ refers to species delineation of Bouvet and Jeanjean [55] and TU refers to Tjernberg and Ursing [20]; [b] *A. grimontii* was recently reclassified as a later synonym of *A. junii* [8]; [c] There is a lack of evidence that *A. indicus* is a different species from *A. guangdongensis* [57]; [d] *A. dijkshoorniae* and *A. lactucae* were published almost concurrently and their descriptions did not include a specific comparison between each other. Based on the rules of priority, *A. dijkshoorniae* should be reclassified as a later heterotypic synonym of *A. lactucae* [58]; [e] There is a lack of evidence that *A. septicus* is a different species from *A. ursingii* [7]; [f] The name *"A. venetianus"* was used before 2009 to designate three marine hydrocarbon-degrading *Acinetobacter* strains, of which strain RAG-1 (ATCC 31012) was used for the production of the bioemulsifier emulsan. However, the name of this taxon was just validly published in 2009 by Vaneechoutte M. and colleagues [54].

* Species delineation based on a single isolate.

1.1.2. Identification and typing: overview of the methods applied to *Acinetobacter* species

"Science never solves a problem without creating ten more."
George Bernard Shaw

For many years, some *Acinetobacter* species were very difficult to identify by the conventional phenotypic tests (based on morphological, physiological and metabolic characteristics) that were used by the majority of routine microbiology laboratories. This identification was particularly difficult within the phenotypically more homogeneous species, like those belonging to the *Acinetobacter calcoaceticus* - *Acinetobacter baumannii* complex (ACB complex) that includes *A. baumannii*, *A. calcoaceticus*, *Acinetobacter pittii*, *Acinetobacter nosocomialis*, *Acinetobacter seifertii* and *Acinetobacter dijkshoorniae* [50]. Thus, for a long time, it was only possible to discriminate these species using genomic tools. However, and since the antibiotic susceptibility and clinical relevance are significantly different among the different *Acinetobacter* species, their exact identification should be included under the routine practice, being important to guide therapy.

Recently, more sophisticated omic methodologies [average nucleotide identity (ANI) determined with whole genome sequencing (WGS), Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF MS) and Fourier transform infrared (FTIR) spectroscopy] have been explored and used for bacterial species identification and typing (Box 1) [59,60], being applied in a wide variety of contexts: in retrospective epidemiological studies; to recognize and track individual strains or epidemic clones (clones with higher or specific pathogenicity); to identify patterns of infection and sources of transmission; to study the population structure and phylogeny of a given species, and also for the detection of outbreaks and reservoirs or sources of human pathogens [61,62].

BOX 1

Glossary of concepts commonly used in bacterial typing.

Phenotyping: discrimination of a bacterial isolate/strain based on the observable characteristics (e.g. morphology of colonies on various culture media, biochemical tests, serology, pathogenicity and antibiotic susceptibility) [63].

Genotyping: refers to the discrimination of bacterial strains based on their genetic content [63].

Fingerprint: specific pattern (e.g. DNA banding pattern; peak patterns) or a set of markers scores (e.g. absorbance values) displayed by an isolate resulting from the application of one or more typing methods, that can be used to assess epidemiological relatedness among bacterial isolates [62].

Reproducibility: ability of a typing method to generate the same results upon repeating testing [64].

Discriminatory power: ability of a typing method to distinguish between epidemiologically unrelated strains.

Sensitivity: proportion of positives that are correctly identified as such [61].

Specificity: proportion of negatives that are correctly identified as such [61].

Typeability: The proportion of strains that are assigned a type by a giving typing method [64].

Taxonomy: The science of naming, describing and classifying organisms, and includes all plants, animals and microorganisms of the world (https://www.cbd.int/gti/taxonomy.shtml) [62].

Phylogeny: evolutionary relationships among members of the same taxon (e.g. species, strains, clones) [62].

Phylogenetic tree: a diagram that depicts the hypothetical evolutionary history of the taxa under study. The points at which lineages split represent ancestor taxa to the descendant taxa appearing at the terminal points of the tree [62].

Species: The basic taxonomic category of bacteria; consists of strains of common origin which are more similar to each other than they are to any other strain [65].

Type strain: pure and well-characterized strain with which the name of the species is permanently associated. The type strain is one of the first specimens of a described species and is marked by a superscript T at the end of its identification number [62,64].

Strain: isolate or group of isolates exhibiting common phenotypic and/or genotypic traits that differ from other isolates of the same species [64].

Isolate: population of microbial cells in pure culture, usually obtained by sub-culturing a single colony from a preliminary isolation plate [66].

Lineage: group of isolates sharing essential characteristics due to a common ancestor. Lineages are generally inferred from a phylogenetic tree [62].

Clone: bacterial isolates descending from a common ancestor and exhibiting identical or closely

similar phenotypic or genotypic traits (even if they have been cultured independently from different sources in different locations and even at different times) [62].

Population: group of organisms of the same species inhabiting a particular environment [67].

Population dynamics: the study of factors that interfere with the variability of bacterial populations over time and space, including the interactions of these two factors [62].

Endemic: The constant presence of diseases or infectious agents within a given geographic area or population group [62].

Epidemic: refers to an increase, often rapid and extensive, in the number of cases involving a particular organism above what is normally expected in that population in that area [62].

Outbreak: temporal increase in the incidence of infection (or colonization) by a certain bacterial species, caused by enhanced transmission of a single epidemic strain (clonal outbreak) or combinations of different strains (multi-strain outbreaks) [64].

Pandemic clone: epidemic clone that has spread over several countries or continents (e.g. ST208 *A. baumannii)* [68–70].

Sequence Type (ST): MLST-based term representing isolates with a particular allelic profile (http://eburst.mlst.net).

Clonal complex (CC): MLST-based term representing a group of related STs sharing alleles at 5/7 or 6/7 of the loci (http://eburst.mlst.net).

Genome: complete genetic information of an organism as encoded in its DNA and/or RNA (http://www.nature.com/subjects/genomics).

Genomics: refers to the study of the full genetic complement of an organism (the genome) (http://www.nature.com/subjects/genomics).

Core genome: set of genes shared by all of the genomes studied (e.g. genomes belonging to the same clone, bacterial species, etc.). These genes are generally involved in essential cellular processes (housekeeping and regulatory functions) [71].

Accessory genome: set of genes shared by some organisms, which are not present in all of the studied organisms (these elements are generally associated with mobile genetic elements) [71].

Pan-genome: refers to the total number of non-redundant genes that are present in a given dataset. It comprises basically three parts: i) core genome, ii) accessory genome, and iii) species-specific or strain-specific genes, which are those genes that are present in a single genome [71].

1.1.2.1. Phenotypic and genotypic methods

Currently, *Acinetobacter* species are defined by both phenotypic and genotypic characteristics, and are represented by type strains deposited in international culture collections. Phenotypic tests are based on colony morphology, colour, odour or other macroscopic features, and also on biochemical traits, such as the ability to use certain substrates (e.g. API 20NE). In addition, commercially platforms (automated and semi-automated) are also available but, *per se*, insufficient and inaccurate [72]. Thus, several genomic methods (based on nucleic acid analysis) have been developed or improved, with several significant advantages [73,74]. DNA-DNA hybridization (DDH) techniques have been used as the "gold standard" for the genomic similarity analysis of pair-wise sets of strains for classification purposes [72], and the amplified fragment length polymorphism (AFLP); the study of ribosomal DNA (e.g. amplified 16S ribosomal DNA restriction analysis-ARDRA); and the phylogenetic analysis based on single or multilocus sequence analysis (MLSA) of housekeeping genes [(mainly 16S rRNA or the intergenic spacer (ITS) region 16S-23S rRNA genes, RNA polymerase β-subunit (*rpoB*), and DNA gyrase subunit B (*gyrB*)]. In addition, some of the clinically relevant *Acinetobacter* species harbour species-specific genes for a class D β-lactamase, whose detection can be used for identification (e.g. $bla_{\text{OXA-51-like}}$ in *A. baumannii*, $bla_{\text{OXA-134-like}}$ in *A. lwoffii*, $bla_{\text{OXA-211-like}}$ in *A. calcoaceticus* and $bla_{\text{OXA-228-like}}$ in *A. bereziniae*) [75]. However, these housekeeping genes may not be sufficiently variable to distinguish all *Acinetobacter* species and some of these methodologies are laborious and available only in reference laboratories, making the identification of *Acinetobacter* species in routine diagnostic laboratories very difficult.

Currently, with the advances in sequencing technologies, the continuing reduction costs and the shortening of the results delivery, microbiology laboratories have increased their access to the whole genome sequencing (WGS), a technique that promises to replace some older tools used to identify and characterize strains at the genomic level. Thus, the whole genome-based average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) are starting to replace DDH for species identification. The ANI is a similarity index calculated between a given pair of genomes that can be applicable to prokaryotic organisms independently of their G+C content. An ANI cut-off score >95% indicates that the comparing genomes belong to the same species, with ANI values ≥ 95% corresponding to the traditional 70% DDH threshold [76].

In what concerns the infra-species level characterization, typing methodologies are mainly used for *A. baumannii* due to its clinical relevance. There is a wide range of epidemiological methods available for researchers and organizations specialized in

different domains, such as medical routine microbiology, infection control management and surveillance, being important that the selected method fit their epidemiological purposes. These methods can be divided in the DNA banding pattern- and DNA sequencing- based methods [74], and also according to their applicability at local scale (e.g. pulsed-field gel electrophoresis, PFGE) or at a large scale (e.g. multilocus sequence typing, MLST) (Table 2).

Despite the emergence of new approaches, PFGE is still considered one important epidemiological tool for *A. baumannii* characterization [77]. The genome is cut by a restriction enzyme (e.g. *Apa*I, *Sma*I), producing a few large fragments that are separated by electrophoresis, periodically changing the direction of an electric field, and allowing the resolution of large fragments up to 1000 kb [74]. The PFGE resulting patterns are usually analysed by computer software programs (e.g. Infoquest from Biorad). The most commonly used similarity cut-off in the prediction of *A. baumannii* putative clones is >87% [77]. Despite the high discriminatory power of PFGE, which is useful in outbreak investigations, it cannot be considered as a method of choice for large scale studies due to genetic events that may occur from one generation to another that may interfere with the patterns generated [78]. Besides, PFGE suffers from several limitations: is technically demanding and time-consuming, some strains are non-typeable and the inter-laboratory reproducibility and comparability is difficult mainly due to the high sensitivity of electrophoresis-based method and the subjective analysis of the results (Table 2) [74].

MLST provides a uniform nomenclature owing to a standard reproducible system and thus is suitable for large-scale epidemiology, being extensively used in the identification and characterization of main clones implicated in the dissemination of multidrug-resistance (MDR) and extensive drug-resistance (XDR) *A. baumannii* strains worldwide [79–81]. It assesses the genetic variation that accumulates slowly within a population by targeting seven housekeeping genes involved in essential metabolic processes. For *A. baumannii* two MLST schemes have been developed: one proposed by Bartual *et al.* [82] (Oxford Database), and other proposed by Diancourt *et al.* [83] (Pasteur database), with some studies pointing for a better discrimination of the first one [84]. Recently, these MLST databases (https://pubmlst.org/abaumannii/) were developed for the Bacterial Isolate Genome Sequence Database (BIGSdb genomics platform) [85] to assist the broader community in elucidating the structure and function of this microorganism. MLST has many advantages that makes it the current gold standard for population structure investigations: its portability and its ability to generate highly standardized, reproducible data that can be stored in a database; and its high discriminatory power in long-term global studies [74].

The recent burst of new high-throughput sequencing technologies was crucial to provide a higher resolution of *A. baumannii* populations, and to explore factors responsible for its global expansion and maintenance. Unlike traditional typing methods, WGS-based approaches decipher the total information present in bacterial genomes, being suitable to be used in different epidemiological contexts, ranging from fine epidemiological [86], to local [87] and global studies through to phylogenetic and population structure studies [87]. Within a small-scale outbreak, WGS can resolve genetic differences between very closely related strains (sometimes indistinguishable by PFGE and MLST) and can enlighten unclear routes of transmission between patients [86]. At the strain level, WGS provides opportunities to explore and identify useful gene contents within bacterial genomes such as antimicrobial resistance determinants including resistance islands, virulence genes and additional informative genome sequences that can also serve for fine epidemiological ends or to explain some exhibited phenotypes [75,88]. Moreover, WGS can also be used to define the set of genes of core and accessory genomes [89] and in phylogenetic analyses of core genomes, either by the development of a cgMLST scheme [90] or by the comparison of single nucleotide polymorphisms (SNPs) across the whole genome [91]. However, the use of WGS in routine clinical laboratories for typing is still costly and requires specific skills for data processing.

Table 2 summarizes the main characteristics, applicability, advantages and limitations of the typing methods frequently used for *A. baumannii* characterization.

TABLE 2. Applicability and main characteristics of the most common epidemiological typing methods for *A. baumannii* (Adapted from [74]).

Molecular typing method	Genetic target	Main steps	Advantages	Limitations	Applications and notes	References
DNA banding-based methods						
RAPD	Whole genome	PCR with arbitrary primers followed by GE	Rapid, easy to use, low cost, acceptable discriminatory power	Lack of inter and intra reproducibility and unportability of results	Screening epidemiological tool at local scale (e.g. outbreaks)	[92]
Rep-PCR	Whole genome, especially sequences located between repetitive sequences	PCR with outwardly primers complementary to repetitive sequences (rep1 and rep2) followed by GE	Rapid, easy to use, high discriminatory power. Automated form: High intra- and inter-laboratory reproducibility, good portability	Manual form: lack of inter and intra reproducibility. DiversiLab: expensive, need of manual extraction and lack of similarity cut-offs provided by manufacturer	DiversiLab: clonal screen at local, large scale, and during population structure studies	[93,94]
Ribotyping	rDNA and flanking regions	DNA restriction (EcoRI or HindIII and HincIII) followed by Southern blot and probe hybridizations	High resolution, reproducibility	Low discriminatory power. Manual form: laborious; Automated form: expensive, need manual correction of its clustering	Identification of *A. baumannii* complex at species, clone (including international clones), and at lesser extent at epidemic strain level	[95,96]
PFGE	Whole genome	DNA restriction by infrequent cutter (*Apa*I, *Sma*I) followed by PFGE	High discriminatory power	Laborious, untypeability of some strains, low resolution for similar fragment size, lack of inter-reproducibility, unportability of results	Useful for outbreak investigation and epidemiological surveillance at local or large-scale level, international databases are widely available.	[77]
AFLP	Whole genome	DNA restriction by 2 enzymes (MseI, EcoRI), followed by selective amplification with Cyc-5EcoRI+A and MseI+C primers (A	Deep resolution of genomic relatedness at different levels (species, clone, strain); high discriminatory	Laborious, expensive, lack of inter-reproducibility	Important tool in *Acinetobacter* taxonomy and gold standard for *A. baumannii* molecular epidemiology (differentiation of the main clonal lineages).	[95]

TABLE 2 - *Continued*

Molecular typing method	Genetic target	Main steps	Advantages	Limitations	Applications and notes	References
		and C selective nucleotides) and GE	power, presence of established database			
MLVA	Genetic polymorphism within VNTR loci	Amplification of VNTR loci followed by GE	Portability, reproducibility, high discriminatory power, amenability to automation, availability of electronic database	Rapid evolution of certain loci, careful interpretation of results	Performing typing at fine scale on very closely related strains, as well as at large scale and population structure studies	[97–100]
DNA sequencing-based methods						
***bla*$_{OXA-51}$-SBT**	*bla*$_{OXA-51}$ gene	Amplification and sequencing of *bla*$_{OXA-51}$	Simple, rapid and low-cost; high accuracy to identify isolates belonging to the 3 major epidemic lineages	Slight variability of the *bla*$_{OXA-51}$ in some *A. baumannii* lineages	Useful for tracking the worldwide epidemic *A. baumannii* lineages	[101]
3-LST	Polymorphism within three loci *ompA*, *csuE*, *bla*$_{OXA-51}$	Sequence analysis of *ompA* (686 bp), *csuE* (449 bp), *bla*$_{OXA-51}$ (693 bp) or multiplex PCR targeting these 3 genes	Rapid, easy, availability of electronic database			[102]
Oxford MLST scheme	Polymorphism within 7 housekeeping genes	Internal sequence analysis of 7 genes (*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, *rpoD*)	Portability, excellent reproducibility, availability of electronic database	Labour, high cost, careful interpretation of recombination bias	Method of choice for large-scale epidemiological studies and for population structure studies; not suitable for local outbreak investigations and surveillance studies; Oxford scheme presents higher discriminatory power than Pasteur scheme.	[82–84]
Pasteur MLST scheme		Internal sequence analysis of 7 genes				

TABLE 2 - *Continued*

Molecular typing method	Genetic target	Main steps	Advantages	Limitations	Applications and notes	References
		(*cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, *rpoB*)				
PCR-ESI/MS	Polymorphism within 9 internal fragments of 6 housekeeping genes	Multilocus PCR (9 primers: 1 primer for each *trpE*, *adk*, *efp* and *ppa* genes, 2 for *mutY*, and 3 for *fumC*) followed by ESI-MS	Rapid, good portability, automated, multifunctional tool	Expensive, limited accessibility in clinical laboratories	Good ability for identification, genotyping at clonal level and at lesser extent at strain level, for population structure studies as well as for determination of antibiotic resistance	[103]
WGS	Whole-genome polymorphism	Whole-genome sequencing and comparative genome analysis	Rapid, automated with high-throughput sequencing techniques	Expensive, limited accessibility in clinical laboratories	Good ability for genotyping at fine scale; provides a complete record of microevolutionary changes.	[89,104]
Accessory genome-based methods						
PCR-based replicon typing scheme	Intrinsic plasmid (19 groups based on their replicase genes homology)	6 PCR multiplexes defining the 19 plasmids homology groups	Easy, cheap, multifunctional tool	Need of validation	Determination of *Acinetobacter* pan plasmidome, circulating plasmids, evolution of resistance genes	[105]

Abbreviations: 3-LST: 3-locus sequence typing; **AFLP:** Amplified fragment length polymorphism; ***bla*$_{\text{OXA-51}}$-SBT:** Single-locus *bla*$_{\text{OXA-51-like}}$ sequence-based typing; **GE:** Gel electrophoresis; **MLST:** Multilocus sequence typing; **MLVA:** Multiple-locus variable number of tandem repeat analysis; **NV:** Not validated; **PCR/ESI-MS:** Multilocus PCR/electrospray ionization mass spectrometry; **PFGE:** Pulse-field gel electrophoresis; **RAPD:** Random amplified polymorphic DNA; **Rep-PCR:** Repetitive sequencing-based PCR; **VNTR:** Variable-number tandem repeat; **WGS:** Whole genome sequencing.

1.1.2.2. Spectroscopic (FTIR) and Spectrometric (MALDI-TOF MS) approaches

Fourier transform infrared (FTIR) spectroscopy and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF MS) are becoming important tools in microbiology for bacterial identification and characterization.

FTIR spectroscopy is a whole-organism fingerprinting method that generates metabolic specific patterns from a bacterial cell (Box 2). It reflects the phenotype of the microorganism under investigation, through the detection of a diversity of biomolecules (lipids, polysaccharides, proteins and nucleic acids), and represents an attractive alternative to the genotyping methods [106].

BOX 2

Principle of FTIR identification of microorganisms.

- FTIR spectra results from the interaction of infrared radiation with the bacterial isolate, which changes the vibrational behaviour of molecules by delivering energy quanta and changing their vibrational and rotational modes. This provides a specific fingerprint that reflects the structure and composition of the whole cell [107]. However, in order to obtain a highly reproducible spectra, is indispensable to follow a strictly standardized protocol for isolates culture (media, time and temperature), sample preparation and spectra acquisition [108].

- Different sampling techniques such as transmittance diffuse reflectance and attenuated total reflectance (ATR) are used in FTIR (Figure 1). In ATR mode, the sample is placed directly onto a crystal of relatively higher refractive index, needing little or no sample preparation.

- The term "Fourier transform infrared spectroscopy" originates from the fact that a Fourier transform is required to convert the raw data (interferogram) into the actual spectrum. Moreover, to obtain a relevant picture of the relationships between spectra and assist in the classification process, several chemometric procedures that use mathematical methods can be applied. Are examples of these the supervised techniques (e.g. partial least squares discriminant analysis - PLSDA), that make use of a priori knowledge of classes to guide in the characterization or classification process, and the unsupervised methods (e.g. principal component analysis - PCA), that try to disclose naturally-occurring groups and structures within the data set without previous knowledge of class assignment [107,108].

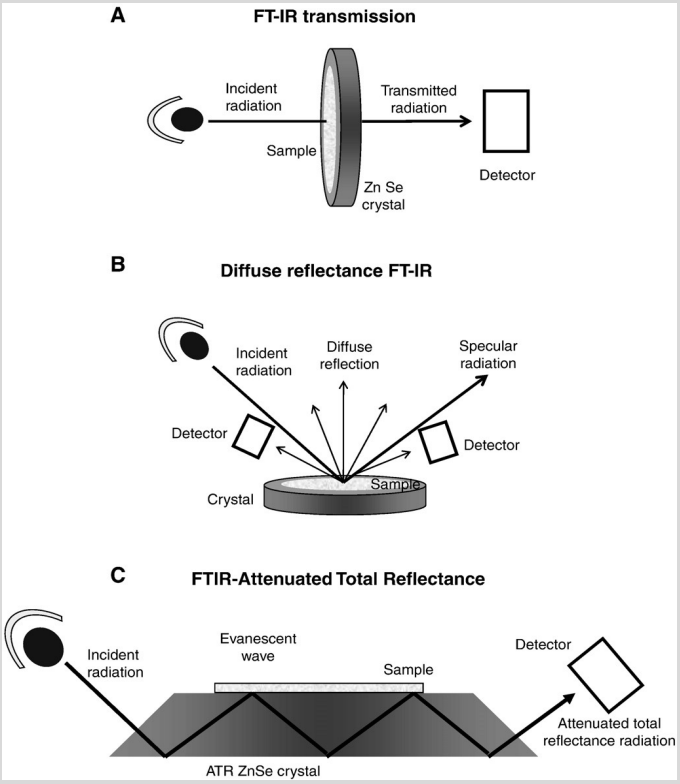


FIGURE 1. Schematic representation of the main spectroscopic methods used by FTIR spectroscopy. (A) FTIR transmission (B) Diffuse reflectance FTIR (C) FTIR-Attenuated Total Reflectance (reprinted with permission from [107]).

This method was introduced into bacteriology in the 1970´s and, since then, has gained growing interest to detect and differentiate several microorganisms [bacteria (e.g. *Staphylococcus* spp., *Enterococcus* spp., *Salmonella* spp. and *Escherichia* spp. strains), yeast (e.g. *Malassezia* species), fungi (e.g. *Candida* species) and algae (e.g. order Sphaeropleales)] at different taxonomic levels, including for species identification [109–117] [111,118–122]. Among *Acinetobacter*, FTIR spectroscopic typing started in 1995 with a collection of *A. baumannii* isolates [123] but, since then, their discriminatory power for the characterization of this genus has been poorly analysed [122,124]. The spectral region more used in these studies corresponds to the phospholipids/DNA/RNA and polysaccharides vibrations (1500-900 cm^{-1}; Figure 2) for being the most discriminatory one [122,125].

FTIR is quick, low-cost, environmentally friendly, requires minimal sample preparation, no consumables or reagents and allows the analysis of small quantities of biomass [118,124].

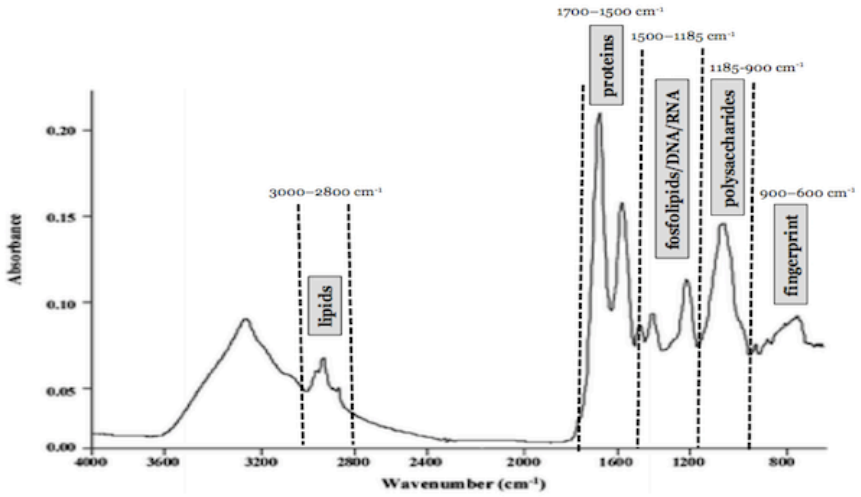


FIGURE 2. Significant regions of bacterial spectra.

MALDI-TOF MS (Box 3) is a high-throughput analytical technique based on the ionization and separation of ions according to mass-to-charge (m/z) ratio, yielding a characteristic fingerprint of a given microbial cell that reflects mostly the content of peptides and small proteins (mainly ribosomal proteins) (Figure 3) [126]. Over the past few years, MALDI-TOF MS has been spotlighted as a powerful tool for the identification, at genus and species level, of a broad spectrum of bacterial species (including Gram-positive and Gram-negative), replacing the traditional biochemical or molecular techniques in many clinical microbiology laboratories worldwide [126,127]. Microbial identification is established by the comparison of the mass spectra obtained with those from known species included in a reference database. Moreover, MALDI-TOF MS has also been used to detect antibiotic resistance (e.g. for the detection of ESBL-producing [128] and carbapenemase-producing strains [129,130]); for environmental bacteriology, food and water quality control, occupational safety, detection and identification of agents of biological warfare, and others [131].

BOX 3

Principle of MALDI-TOF MS identification of microorganisms.

- The sample is deposited on a metal plate and embedded in a matrix that crystallizes the analytes, being then bombarded by brief laser pulses that achieve the ionization (MALDI-TOF MS) by proton transfer from the matrix, which results in positively charged analytes;
- The desorbed ions are then accelerated by an electrostatic field and directed in the flight tube, in which they are separated according to their time of flight (TOF) in the flight tube (with a high vacuum generated by a pump);

♦ Ions are detected at the exit of the flight tube, where a software generates a mass spectrum.

♦ Microbial identification is established by the comparison of the mass fingerprint obtained (characteristic for each microorganism), directly from the colony or after using adequate extraction protocols, with those from known species included in a reference library [126,132].

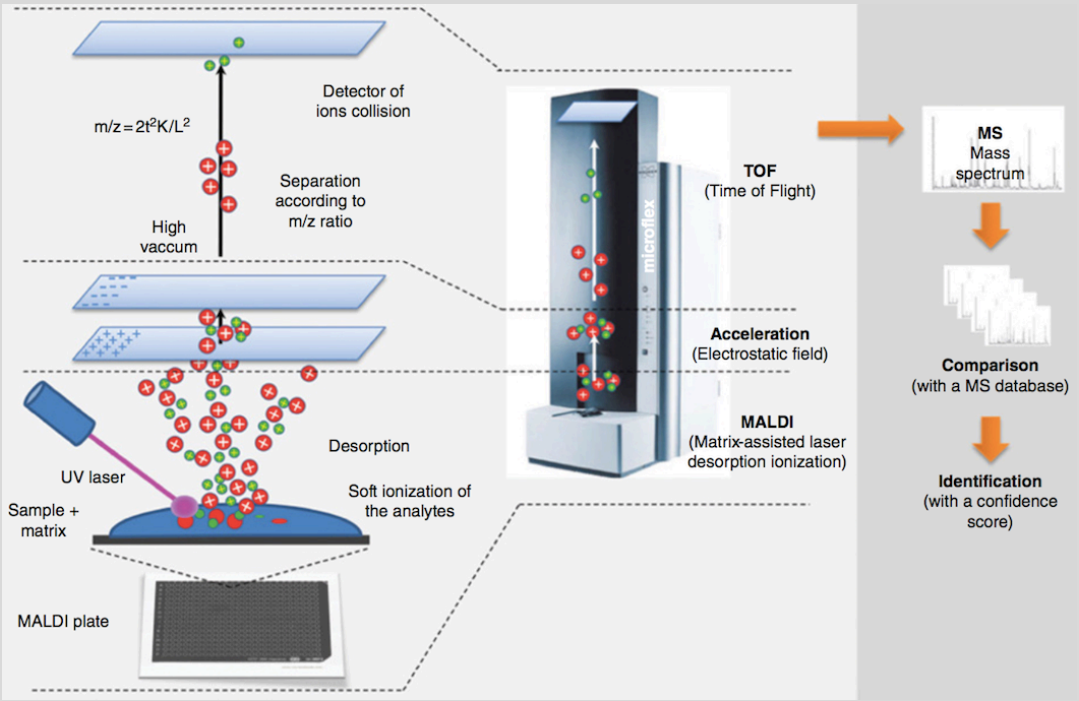


FIGURE 3. Principle of **MALDI-TOF MS** (reprinted with permission from [132]).

This method is easy to use, cost-effective, accurate, sensitive and rapid, with potential to enhance the implementation of infection control measures. A number of examples of the potential usefulness of this proteomic technology for the identification of microorganisms have been published [133,134]. However, the majority of these studies presented several flaws, since the current automatic MALDI-TOF MS databases, available for routine laboratories, do not cover all *Acinetobacter* species, being needed a chemometric analysis for an accurate species identification. Moreover, although many attempts have been made in order to adapt this technology also for the identification at the strain level [135], the great majority of the results obtained are still controversial, with many inconsistencies being observed even for the same species [136,137]. These could be due to the wide diversity of analysis workflows (e.g. variations in growth conditions, extraction methods, matrix, mass spectrometers and bioinformatics tools used for data analysis) and also because the closely related strains express many similar, if not the same, proteins [61,138]. Thus, one of the next challenges in the development and

implementation of MALDI-TOF MS for typing in the routine diagnostic workflow of clinical laboratories will be to standardize the procedures for cell culturing/preparation and data analysis (with a user-friendly software), in order to improve its inter-laboratory reproducibility, discriminatory power, robustness, resolution and portability [61].

1.1.3. *Acinetobacter* ecology

"I fear all we have done is to awaken a sleeping giant and fill him with a terrible resolve."

Isoroku Yamamoto

Acinetobacter species comprise a broad group of biochemically and physiologically versatile bacteria that are ubiquitous in nature and can be found in different ecological niches, such as environment, food, animals and humans. Thus, several *Acinetobacter* species appear in the **environment** (e.g. *A. baylyi*, *A. towneri* and *A. gerneri* from activated sludge, sewage and wastewater; *A. seohaensis* from seawater; *A. soli* from forest soil and *A. johnsonii* from water); in the **food chain** (e.g. *A. calcoaceticus*, *A. pittii*, *A. lwoffii*, *A. bereziniae* and *A. soli* from vegetables, raw meat, cheese and milk); in **animals** (e.g. *A. variabilis* from rectal cow samples; *A. gandensis* from horse and cattle, *A. radioresistens* and *A. schindleri* from head lice); and in **humans** (e.g. *A. lwoffii*, *A. johnsonii*, *A. haemolyticus*, *A. radioresistens*, *A. pittii* and *A. junii* as colonizers of skin and *A. johnsonii* and *A. pittii* from faecal samples) (Table 3) [139]. The ability to adapt to many ecological niches has led some authors to consider these bacteria as "microbial weeds" [140]. Despite the several descriptions of *Acinetobacter* spp. in the environment, animals and healthy humans, the precise environmental reservoirs are unknown. Recently, it has been shown that the environment could also constitute a potential reservoir for *Acinetobacter* spp. resistant isolates, being carbapenemase and extended-spectrum β-lactamase (ESBL) producing strains isolated from, for example, soil of farms [141], municipal wastewater [142], and in tap water and rivers [143,144]. Thus, further studies outside hospital units are of outmost importance for a precise definition of reservoirs and for elucidating the emergence of infections by MDR *Acinetobacter* spp..

In opposition to the other species, *A. baumannii* is mostly reported as an infection agent in the hospital setting, particularly in Intensive Care Units (ICUs), despite community-acquired infections may also occur [145,146]. Increasing evidence reveal a similar behaviour in the veterinary practice [143,147,148]. Moreover, many descriptions of occasional isolates of *A. baumannii* from non-human sources, such as animals, lice, vegetables, aquaculture and soils were also published [149–152]

1.1.4. Acinetobacter spp.: the emergence of the pathogen

"In the field of biological weapons, there is almost no prospect of detecting a pathogen until it has been used in an attack."

Barton Gellman

For many years, organisms belonging to the genus *Acinetobacter* were regarded as low-virulence bacteria. However, some species have become successful pathogens in the 1970s (with the introduction of broad-spectrum antibiotics for *Enterobacteriaceae* treatment), with *A. baumannii* being now considered, by the World Health Organization (WHO) (http://www.who.int/en/), as one of the most serious ESKAPE organisms (***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****. baumannii*, ***P****seudomonas aeruginosa* and ***E****nterobacter* species), due to the emergence of drug-resistant strains and an increased number of infections (with high mortality rates) [153,154]. Although *A. baumannii* is undoubtedly the main species associated with nosocomial infections, it is of note the fact that in recent years, we have assisted to increasing reports of infections (including outbreaks) and/or colonization caused by non-*baumannii Acinetobacter* species [155]. Species like *A. pittii* [156], *A. bereziniae* [157], *A. ursingii* [158], *A. johnsonii* [159], *A. junii* [160], among others, have been entering in the clinical setting. Moreover, these species can also acquire and act as a source (silent in some cases) of resistance genes, contributing for their dissemination among different *Acinetobacter* species [161,162].

The majority of infections caused by *A. baumannii* are mostly hospital-acquired, commonly in the ICUs, with the major risk factors including recent exposure to antimicrobial agents (in particular carbapenems), presence of central venous or urinary catheters, mechanical ventilation, severity of illness, underlying malignancy, history of transplant, older age, prolonged hospital stays, or larger hospital size. In addition, this organism was also found colonizing inpatients from long-term care facilities (LTCFs) and as a cause of infections acquired in the community and in wounded military personnel [163,164]. The associated infections include, but are not limited to, pneumonia (both hospital and community-acquired), bacteraemia, endocarditis, skin and soft tissue infections, urinary tract infections and meningitis. In most cases, it is thought that the acquisition and spread of this organism inside the clinical settings is associated with contaminated equipment, contact with colonized patients and/or healthcare workers [165,166]. Healthcare workers, besides being considered important vehicles for infections transmission, could also be affected by infectious agents, e.g., by contact with patients, respiratory droplets, airborne spread, among others [167]. This risk may be enhanced by

factors such as the age or the presence of chronic diseases like diabetes, workload, overcrowded hospitals, and the pathogenic potential of the microorganism. To date, a case of a life-threatening pneumonia resulting in septic shock due to an *A. baumannii* transmission from a patient to a healthcare worker was reported [168].

1.1.5. Overview of virulence features and antimicrobial resistance among *Acinetobacter*

"With increasing resistance even to last-line antibiotics we face a frightening future where routine surgery, childbirth, pneumonia and even skin infections could once again become life threatening."

Vytenis Andriukaitis

Important *Acinetobacter* spp. features, with emphasis on *A. baumannii* will be addressed in this section, in order to understand their clinical relevance and evolution towards MDR phenotypes.

1.1.5.1. Virulence determinants

Compared to other Gram-negative pathogens, relatively few virulence factors have been identified in *Acinetobacter*. Moreover, most known virulence factors of *A. baumannii* are found in its core genome and are also present in other *Acinetobacter* species, thus, in this section, we focused only on *A. baumannii* [169]. The recent sequencing of several complete genomes of this species, together with the use of animal models, have been crucial for shedding light on how this pathogen persists in the environment, interacts with host cells and causes host cell damage, although there are undoubtedly numerous additional factors that have yet to be identified [170].

Table 3 summarizes the most common virulence factors identified, until now, in *A. baumannii*, and their main role in this species pathogenesis.

TABLE 3. Virulence factors identified in *A. baumannii* and their proposed role in pathogenesis. Adapted from [168,170].

Virulence factor	Description/Examples	Proposed role in pathogenesis	References
Porins and efflux proteins	OmpA, Omp22, Omp33-36, CarO, OprD-like and AceI	Adherence and invasion, induction of apoptosis, serum resistance, surface motility, biofilm formation, persistence and chlorhexidine resistance	[168,171,172]
Phospholipases	Phospholipases- A (PLA), -C (PLC) and -D (PLD)	Serum resistance, invasion, *in vivo* survival	[173,174]

Virulence factor	Description/Examples	Proposed role in pathogenesis	References
Outer membrane vesicles (OMV)		Delivery of virulence factors, horizontal gene transfer	[168,175–177]
Metal acquisition systems	Iron acquisition systems: enterolisin E, heme oxygenase, and siderophores like acinetobactin and fimsbactin A-F; Zinc acquisition system: ZnuABC, His-Zn complex and calprotectin; Manganese acquisition system: MumC and MumT	*In vivo* survival, persistence, killing of host cells	[178–184]
Capsular polysaccharides (KL)	KL variants (e.g. KL1, KL2, KL4)	Growth in serum, survival in tissue infection, biofilm formation; evasion of host immune response, survival to desiccation	[168,185,186]
Lipopolysaccharides (LOS)	LpsB, LpxC	Serum resistance, survival in tissue infection, evasion of the host immune response, antibiotic resistance by target alteration	[168,170,187]
Protein secretion systems	Type II protein secretion system Type VI protein secretion system Type I secretion system (Bap_{Ab}) Type V protein secretion system	*In vivo* survival, export of effector proteins (e.g. LipA and CpaA); Killing of competing bacteria, host colonization Biofilm formation, adherence	[168,188–191]
Photoreceptors	BlsA	Motility, biofilm formation, killing ability	[192,193]
Pili systems	Type I pili - Csu pili Type IV pili - Pilin subunit (e.g. fimbrial protein PilA) GacSA system - *csu* gene expression	Adherence, surface-associated and twitching motility, biofilm formation, horizontal gene transfer	[194,195]

The most well-characterized virulence factor identified to date in *A. baumannii* is probably the outer membrane protein A (OmpA), a porin that has been associated, *in vivo*, with pneumonia, bacteraemia and host cells death [196] and with a variety of interesting

biological properties identified in *in vitro* model systems [168]. OmpA interacts with peptidoglycan and has been shown that binds to host epithelia, target mitochondria, translocate to the nucleus, and induces cell death by promoting the release of pro-apoptotic molecules, such as cytochrome C and apoptosis-inducing factor. Moreover, this porin also enhances surface motility, adherence, biofilm formation and resistance to the complement-mediated killing [197], being also considered a slow porin for β-lactams [198] and associated with antimicrobial resistance to chloramphenicol and nalidixic acid [171]. The low permeability of this porin, together with the presence of constitutive β-lactamases and multidrug efflux pumps, appears to be essential for the high levels of intrinsic resistance of *A. baumannii* to a high number of antibiotics [198]. OmpA also regulates biogenesis of outer membrane vesicles (OMVs) by controlling their production and protein composition [199].

Other Omps have also been associated with cytotoxicity (and antibiotic resistance) in *A. baumannii*, although on a smaller scale. Some examples include the 33- to 36-kDa Omp protein (Omp33-36) and the carbapenem-associated outer membrane protein (CarO) and OprD-like (Table 2) [171,172].

Besides OmpA, the *A. baumannii* envelope is associated with many other factors that contribute to pathogenicity, like capsular polysaccharides (KL), glycosylated proteins, lipopolysaccharide (LOS) [184], and peptidoglycan. The KL (Box 4, Figure 4) is an extraordinary virulence factor, essential for bacterial adhesion, host colonization, survival in human serum, and also for enhancing resistance to phagocytosis, disinfection and desiccation [168,200]. This structure can even be considered the main virulence factor of *A. baumannii* as strains lacking the capsule are avirulent and readily killed by complement [201]. All KL gene clusters described for *A. baumannii* have similar general genetic organization with differences found predominately in the central sugars region. In other species, like in *K. pneumoniae*, capsular types are related to the severity of infection [186]. Although the knowledge on the prevalence of specific capsular types in each *A. baumannii*-related diseases could be crucial for disease control and prevention (e.g. for the development of capsule-based vaccines), little is known to date for this species.

BOX 4

A. baumannii capsule biosynthesis.

KL: capsular polysaccharide; long-chain polymer composed of oligosaccharide repeats synthesized under the direction of genes clustered at the chromosomal KL locus [located between lipid II flippase (*mviN*) and L-lactate dehydrogenase (*lldD*) genes] [185].

Synthesis of capsule begins with the synthesis of the repeat unit in the cytoplasm. A pre-

formed sugar precursor is linked to the undecaprenyl phosphate (**UndP**) lipid carrier by an initiating transferase (**Itr**) and then, substrate-specific glycosyltransferases (**Gtr**) sequentially link sugars to complete the oligosaccharide, which is translocated across the membrane by the **Wzx** flippase. The **Wzy** polymerase links the capsular units together to form the KL, which is then exported from the periplasm to the cell surface by **Wza**, **Wzb** and **Wzc** [185].

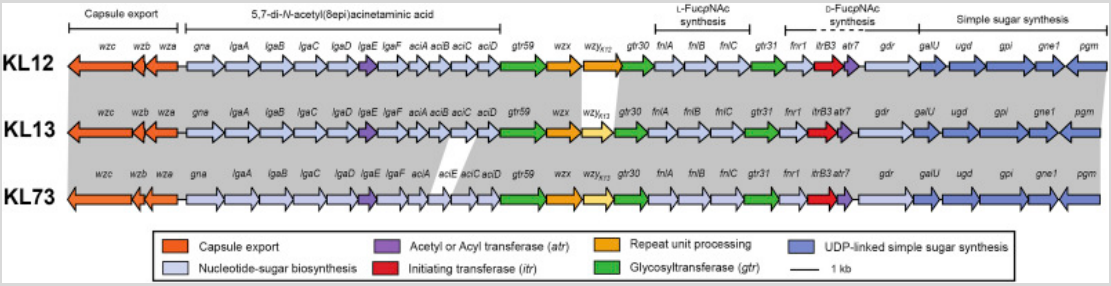


FIGURE 4. Examples of *A. baumannii* capsule biosynthesis gene clusters. Colour scheme shown below corresponds to the function of the gene products, and functions of enzymes encoded by gene modules are shown above. Reprinted with permission from [202].

The lipopolysaccharide (LPS), the major component of the outer membrane leaflet and a hallmark of gram-negative bacteria, in *Acinetobacter* presents an important structural difference, lacking the O antigen. Thus, is called lipooligosacharide (LOS), being also an important virulence factor. The role of LOS in *A. baumannii* pathogenesis was investigated using a mutant lacking the LpsB glycotransferase that results in a highly truncated LOS glycoform showing decreased resistance to human serum and decreased bacterial survival in a rat model of soft tissue infection [187]. Moreover, many studies have shown that modifications in LOS decrease the susceptibility of *A. baumannii* to many clinical important antibiotics, such as colistin, reflecting an important role for the surface carbohydrate residues of LOS in virulence and survival [168].

Bacterial phospholipases are lipolytic enzymes essential for phospholipid metabolism. Degradation of phospholipids affects the stability of host cell membranes, and the cleaved head group can interfere with cellular signalling by generating second messengers (e.g. phosphatidic acid), that results in changes in the host immune response [173]. In *A. baumannii*, phospholipases C (cleave the phosphorylated head group from the phospholipid) and D (cleave off only the head group of phospholipid after its release by phospholipase C) have been identified as virulence factors, being responsible for survival in human serum and epithelial cell invasion [174,203].

Outer membrane vesicles (OMVs) are spherical vesicles (20 to 300 nanometers in size), whose formation is triggered by the reduction in the cross-linking between peptidoglycan and the outer membrane [175]. They are secreted by the outer membrane of various Gram-negative bacteria, including *Acinetobacter* species [188], and are composed of LOS, outer membrane and periplasmic proteins, phospholipids and DNA, or RNA [168]. Stresses such as high temperature, or presence of antibiotics seem to increase OMV formation [204]. OMVs are recognized as delivery vehicles for bacterial effectors to host cells allowing inter- and intra-species communication and strengthening the interaction with the host [204]. In *A. baumannii* they were originally identified as the secretion platform for OmpA but this species also secrete OMVs containing other virulence factors, including phospholipases, proteases and catalase (which induce cellular damage or innate immune responses) and toxins [168,188]. Interestingly, some works have reported other functions for *A. baumannii* OMVs, related with the spread of antibiotic resistance by horizontal transfer of the OXA-24, OXA-58 and NDM-1 carbapenemase genes [176,177,205]. OMVs also participate in quorum sensing, transfer of nutrients, as well as adsorption of antibiotic peptides for the survival of bacterial population [206,207]. It has also been demonstrated that OMVs can deliver bacterial proteins directly to the host cell cytoplasm via fusion of the OMV with lipid rafts on the host cell membrane, which suggests that one function of OMVs may be the transport of bacterial products over long distances [206].

Siderophores are high-affinity iron-chelating compounds produced by bacteria to overcome iron limitation, since this metal is one of the essential nutrients for host and bacteria but it is rarely found in a free form [178]. Siderophores are classified into three major groups: catecholates, hydroxamates and carboxylates, based in the ligands that are used to chelate the ferric iron [179]. Acinetobactin, a catechol-hydroxamate siderophore composed of 2,3-dihydroxybenzoic acid (DHBA), L-threonine and N-hydroxyhistamine, is the best-characterized siderophore from *A. baumannii* [69]. The gene cluster responsible for acinetobactin production and transport is composed of three systems: *basABCDEFGHIJ* for acinetobactin biosynthesis, *barAB* for acinetobactin release, via a efflux system of the ATP-binding cassette (ABC) transporters superfamily, and *bauABCDEF* for movement of ferric-acinetobactin complexes into bacterial cells via a receptor [180]. Under iron-limited conditions the genes involved in the biosynthesis and transport of acinetobactin are usually significantly up-regulated [195]. Moreover, the acinetobactin production/expression occurs more frequently in MDR/XDR *A. baumannii* isolates [69]. The concentration of extracellular free iron is also a factor that affects the amount of biofilms formed on surfaces. In fact, *A. baumannii* clinical isolates showed a

significant reduction in adhesiveness and biofilm formation ability on biotic and abiotic surfaces, when grown in the presence of an iron-chelating agent [208].

A. baumannii has a high capacity to survive and prosper in the harsh hospital environment, mostly due to its ability to interact, adhere and colonize different types of surfaces, including desiccated abiotic ones (e.g. medical equipment like catheters), as well as biotic surfaces (e.g. human epithelial cells) [170]. Although the desiccation resistance is multifactorial and not yet fully defined, a few studies point out for the importance of capsular polysaccharides for survival during periods of desiccation [200]. Moreover, and since desiccation-rehydration causes various DNA lesions and induces oxidative stress, *A. baumannii* relies on the protective role of the RecA protein (an enzyme required for homologous recombination and recombination repair) and also substantially upregulates proteins associated with detoxifying reactive oxygen species [209].

Generally, the adherence of *A. baumannii* to biotic and abiotic surfaces results in the development of biofilms, which are complex multicellular three-dimensional structures with cells in intimate contact with each other and encased in an extra-cellular matrix that can be comprised of carbohydrates, nucleic acids, proteins, and other macromolecules [210]. Adhesiveness and biofilm formation has been linked to increased antimicrobial resistance, resistance to desiccation and disinfection, and, therefore, represents an important virulence factor [211]. A number of environmental conditions and *A. baumannii* gene products have been shown to play a role in biofilm formation and adherence on both abiotic and biotic surfaces. For the initial steps of bacterial attachment and microcolony formation are required pili-like structures whose production is mediated by the CsuA/BABCDE usher-chaperone assembly system [regulated by a two-component system constituted by the sensor kinase (*bfmS* gene) and the response regulator (*bfmR* gene)] [212]. Moreover, a second two-component system termed GacSA has been shown to moderately control *csu* gene expression and thus indirectly biofilm formation [213].

The bacterial adhesin Bap_{Ab} (biofilm-associated protein), expressed on the cell surface and conserved among different clinical isolates, appears to be needed for cell-to-cell interactions and intercellular adhesion that support biofilm development and maturation [211]. The two latter processes also depend on the capacity of *A. baumannii* clinical isolates to produce and secrete poly-β-1-6-N-acetylglucosamine (PNAG). This major component of the biofilm exopolysaccharide matrix (that provides adhesion between bacterial cells) is encoded by a cluster of four genes (*pgaABCD*), produced by almost all clinical strains, being critical for the grow and formation of fully developed biofilms [214]. Several surface proteins, like OmpA, are also involved in the process,

contributing to the attachment of cells and development of biofilms in biotic (e.g. human alveolar epithelial cells) and abiotic (e.g. plastic) surfaces [211].

Light, in particular blue light, seems to affect several aspects of *A. baumannii* physiology such as motility, virulence and biofilm production. In what concerns biofilms, blue light seems to inhibit their formation. This response is mediated by the BlsA photoreceptor protein, which contains a N-terminal blue-light sensing-using flavin (BLUF) domain [192]. The mechanisms by which BlsA transduces the light signal and controls gene expression are not yet well known. However, it has been demonstrated that the diverse transcription of *blsA* gene at 28ºC and 37ºC differentially affects the response of *A. baumannii* biofilm to the light. Ethanol has also been reported to affect biofilm formation and motility on abiotic surfaces, enhancing the first and decreasing the second [215]. Moreover, physiological concentrations of ethanol found in the bloodstream of individuals with a history of alcohol use disorder sufficiently impair phagocytosis and thus elimination of *A. baumannii* [216]. Both clinical and environmental isolates of *Acinetobacter* spp. were reported to produce quorum-sensing (QS) signal molecules (e.g. N-acyl-homoserine lactone) by which bacteria control adhesiveness, biofilm formation and activate adaptations in response to nutrient conditions and cell population density [217,218].

1.1.5.2. Antimicrobial resistance

Since their introduction into medicine about 70 years ago, antibiotics are arguably the most powerful drugs in medicine. However, their success also threatens to be their downfall [219]. Antimicrobial resistance (see Box 5 to review concepts about this topic) is considered one of the major threats to human health by several world health entities, such as the WHO and the European Centre for Disease Prevention and Control (ECDC) [220]. This grim scenario results from the extraordinary adaptation of bacteria to selective pressures, mainly caused by the intensive use of antibiotics [not only in human medicine but also in non-human applications (e.g. in animal husbandry)], as well as the greater movement of people and the increased industrialization (Figure 5) [153,219,221,222]. In fact, several classes of antimicrobials may accumulate in diverse environments/hosts under non-lethal (sub-inhibitory) concentrations, which may provide a constant selection pressure for maintenance of particular pathogenic bacteria, such as *Acinetobacter* spp. strains or clones carrying antibiotic resistance genes and other adaptive features associated with survival or improved host and environmental colonization ability [221,222].

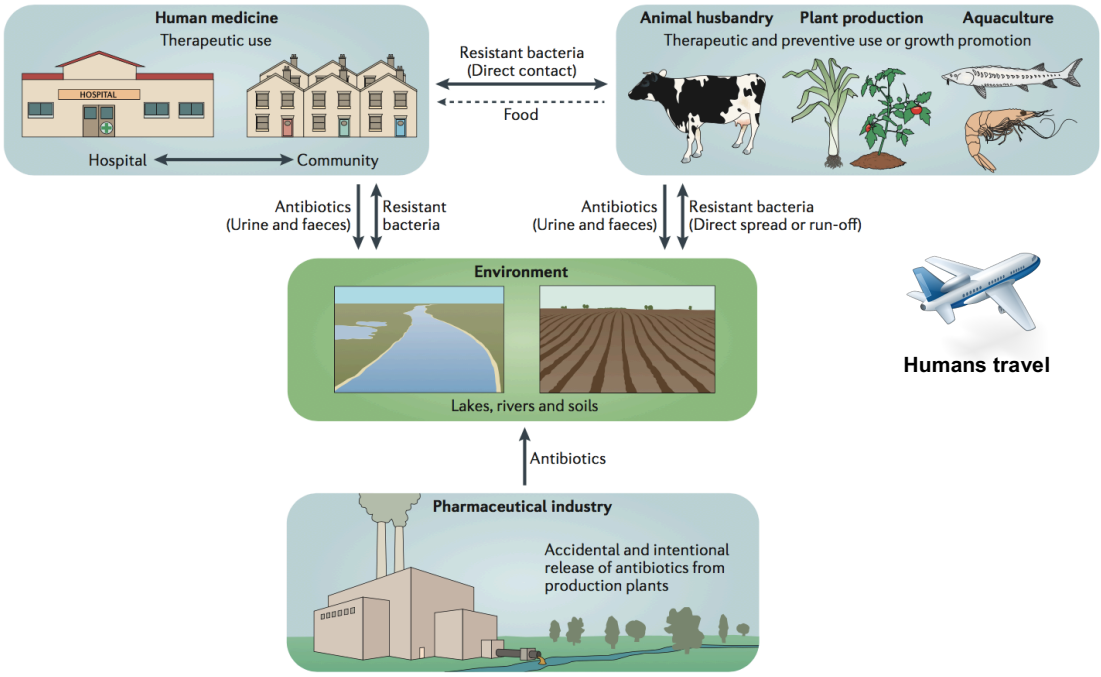


FIGURE 5. Overview of antibiotics cycle among different environments, such as clinical setting, agriculture, aquaculture, pharmaceutical industry and the wider environment (adapted from [222]).

BOX 5

Glossary of concepts related with antibiotic resistance in *Acinetobacter* spp.

The susceptibility of a microorganism to a particular antibiotic can be quantified by phenotypic tests such as the determination of the minimum inhibitory concentration (**MIC**), which is the lowest concentration of an antimicrobial agent that completely inhibits the growth of bacterial cells. The use of **clinical breakpoints** (calculated based on MIC distributions, clinical outcome data, accepted dosing and pharmacokinetic/pharmacodynamics data) is useful to define the **clinical resistance**, used by clinicians to access the likelihood of therapeutic outcome in human patients (Figure 6). Thus, based on clinical breakpoints, the isolate is defined as: **resistant** (level of antimicrobial activity associated with a high likelihood of therapeutic failure), **intermediate** (level of antimicrobial activity associated with uncertain therapeutic effect) or **susceptible** (level of antimicrobial activity associated with a high likelihood of therapeutic success), according to annual published guidelines and recommendations such as those published by the Clinical & Laboratory Standards Institute (CLSI - http://clsi.org/), the European Committee on Antimicrobial Susceptibility Testing (EUCAST - http://mic.eucast.org/Eucast2/) or other national organisms [e.g. British Society for Antimicrobial Chemotherapy (BSAC - http://www.bsac.org.uk)].

Epidemiological resistance is defined using **epidemiological cut-offs (ECOFFs) values** (Figure 6). ECOFFs are the MIC values that correspond to the upper-limit of the wild-type population (population without any acquired resistance mechanism to a particular antibiotic) of a

bacterial species. ECOFFs can be applied to distinguish within a particular species the strains with (**non-wild-type**) or without (**wild-type**) resistance mechanisms (https://mic.eucast.org/Eucast2/SearchController/search.jsp?action=init).

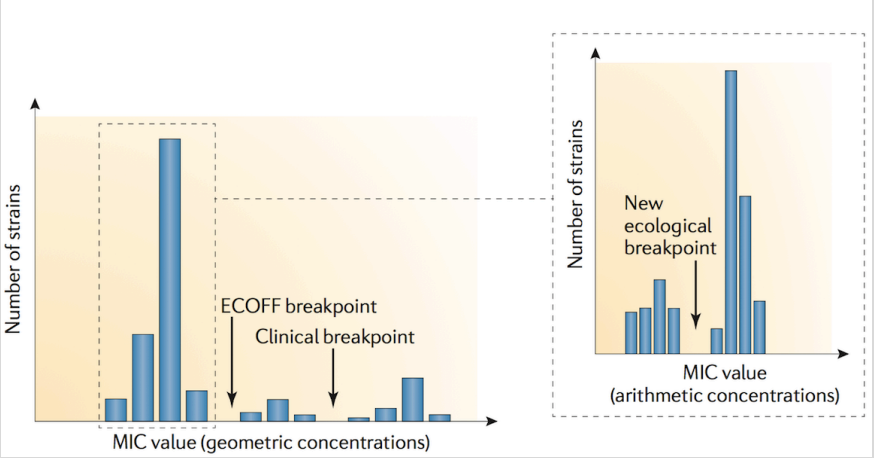


FIGURE 6. Clinical and epidemiological breakpoints of susceptibility to antibiotics (reprinted with permission from reference [223]).

A bacterial isolate is considered non-susceptible to an antimicrobial agent if results resistant or intermediate when using clinical breakpoints as interpretive criteria, and not epidemiological cut-offs. Only acquired antimicrobial resistance was taken into consideration in creating definitions for multidrug-resistant (**MDR**), extensively drug-resistant (**XDR**) and pandrug-resistant (**PDR**) isolates [224]. The criteria for defining MDR, XDR and PDR in *Acinetobacter* spp. according to an international expert proposal to standardise definitions for acquired resistance (Figure 7) [224] is:

- **MDR:** non-susceptibility to ≥1 agent in ≥3 antimicrobial categories.
- **XDR:** non-susceptibility to ≥1 agent in all but ≤2 antimicrobial categories.
- **PDR:** non-susceptibility to all antimicrobial agents.

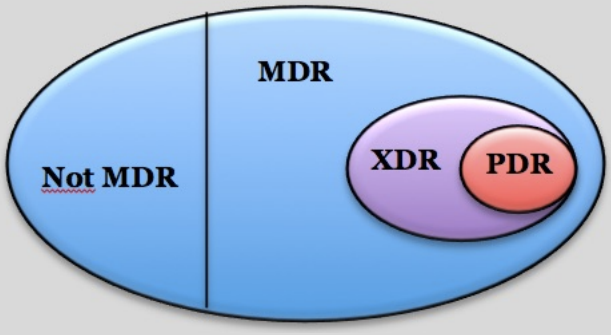


FIGURE 7. Diagram showing the relationship of MDR, XDR and PDR to each other. Adapted from [224].

As previously mentioned, among *Acinetobacter* species, *A. baumannii* has become one of the most successful pathogens in modern healthcare, partly because of its natural reduced susceptibility to antibiotics and amazing ability to acquire new resistance determinants. Antimicrobial resistance among *Acinetobacter* vary with species, the type of antibiotic and geographical location, and is either **intrinsic**, explained by the low permeability of the outer membrane, the constitutive expression of some efflux pumps and the presence of some intrinsic β-lactamases or **acquired**, due to the presence of β-lactamases, aminoglycoside-modifying enzymes, efflux pumps, permeability defects and modifications on target sites [167,168]. The accumulation of these resistance mechanisms has progressively increased since the 1970s, at which time the vast majority of strains were sensitive to commonly used antibiotics. Nowadays, often in several countries, only a small number of therapeutic options are available to treat *Acinetobacter* infections, with descriptions of resistance to all antibiotics also reported [225].

Combined resistance to fluoroquinolones, aminoglycosides and carbapenems was the most frequently reported resistance phenotype in 2016, accounting for almost half of the reported isolates in European countries (Figure 8). This is an indication of seriously limited options for the treatment of patients infected with *Acinetobacter* spp. [220].

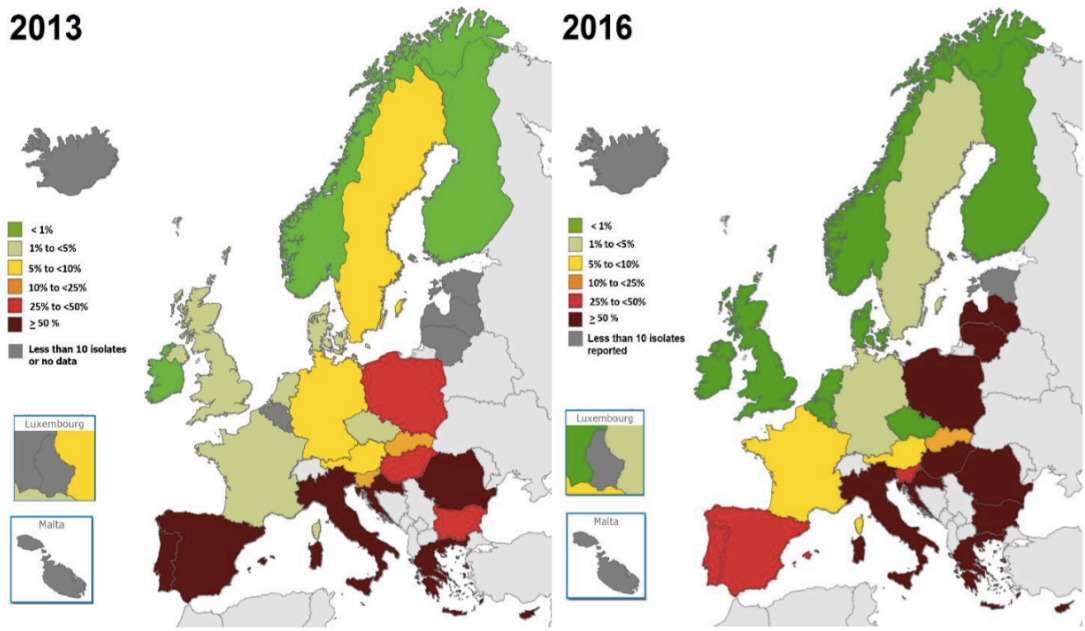


FIGURE 8. Percentage of *Acinetobacter* spp. invasive isolates with combined resistance to fluoroquinolones, aminoglycosides and carbapenems, European Union/European Economic Area, 2013 (left), 2016 (right) [220]. The antibiotic resistance in this bacterium shows large variations across Europe, with generally high resistance percentages reported from the Baltic countries and southern/south-eastern Europe.

In the next section will be summarized the major resistance mechanisms for the different classes of antibiotics that have been identified in *Acinetobacter* spp. (Table 4), with special details for resistance to β-lactams.

1.1.5.2.1. Resistance to β-lactams

β-lactams (see Box 6 for details) are the most widely used bactericidal antibiotics in clinical practice worldwide, with carbapenems being the main therapeutic option for the treatment of infections caused by *Acinetobacter* isolates. However, over the last two decades, resistance to this class of antibiotics has been extensively reported, becoming a serious public health problem.

BOX 6

Mechanisms of action, classification and spectrum of activity of β-lactam antibiotics

Mechanisms of action: β-lactam antibiotics are characterized by the presence of a β-lactam ring in their core structure. Their targets are the bacterial penicillin binding proteins (**PBPs**) (e.g. transpeptidases), which are involved in the biosynthesis of peptidoglycan layer, the main component of the bacterial cell wall. The β-lactam ring binds to these different PBPs, rendering them unable to perform their role and leading to bacterial death by osmotic instability or autolysis (Figure 9). For these reasons, β-lactams are considered **bactericidal** antibiotics.

Classification: According to their chemical structure, β-lactams are divided into four families: 1) **penicillins** (e.g. amoxicillin, ampicillin, ticarcillin, piperacillin, mecillinam); 2) **cephalosporins** (1st-5th generation, e.g. ceftriaxone, ceftazidime, cefotaxime, cefepime, ceftaroline); 3) **carbapenems** (e.g. imipenem, ertapenem, meropenem, doripenem); and 4) **monobactams** (e.g. aztreonam) (Figure 10).

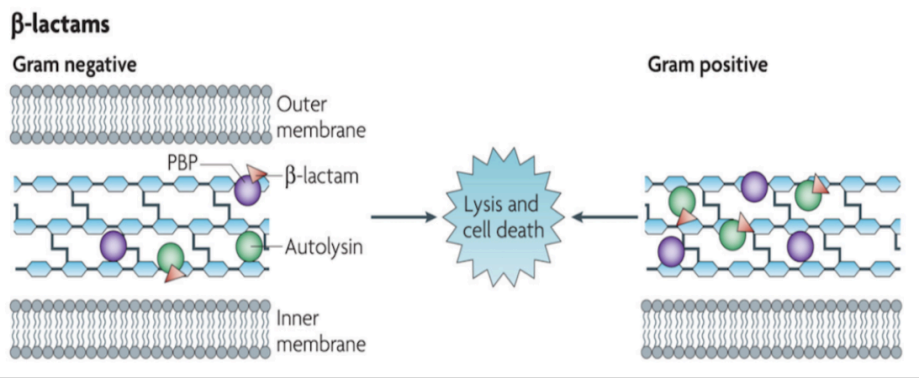


FIGURE 9. β-lactams target interactions and associated cell death mechanisms (reprinted with permission from [226]).

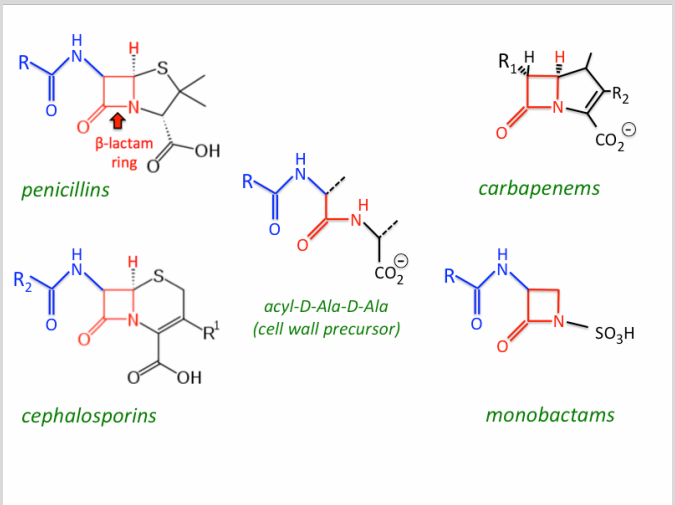


FIGURE 10. Chemical structure of β-lactams (reprinted from http://tmedweb.tulane.edu/pharmwiki/doku.php/antibiotic_pharmacology).

Acquired resistance to β-lactams in *Acinetobacter* spp. can occur due to **enzymatic** (β-lactamases) or **non-enzymatic** mechanisms (loss of or decrease in the expression of porins and/or increased expression of efflux pumps) (Table 4). Inactivation of β-lactams by β-lactamases is a major antibiotic resistance mechanism in *Acinetobacter* spp. and, based on sequence homology, these enzymes are grouped into molecular classes, A, B, C and D. Classes A, C and D are serine-dependent enzymes with a serine moiety at the active site, whereas class B enzymes require divalent metal cations as cofactors (e.g. Zn^{2+}), being designated metallo-β-lactamases (MBLs). Most of these enzymes are associated with mobile or mobilizable genetic elements, such as insertion sequences (ISs) and integrons (see section 1.1.5.2.3.1 for more details), which could lead to enzyme production and dissemination [168,227].

❖ Enzymatic mechanisms:

Class A β-lactamases: A number of class A β-lactamases, including TEM, SHV, GES, CTX-M, SCO, PER, VEB, KPC and CARB have been identified in *A. baumannii* (Table 4). Class A β-lactamases inhibited by clavulanate hydrolyse penicillins and cephalosporins more efficiently than carbapenems, except for some variants of KPC and GES enzymes [228]. Some of these enzymes (e.g. TEM-1, CARB-4 and SCO-1) are narrow-spectrum β-lactamases, whereas others (e.g. PER-1, TEM-92, CARB-10, SHV-5, PER-2, CTX-M-2, CTX-M-15, VEB-1, GES-14 and PER-7) are ESBLs which in addition were detected in non-*baumannii* isolates (e.g. PER-1 and SCO-1) [229]. GES and KPC carbapenemases have

been observed in *A. baumannii* mainly from Mediterranean and Middle East countries [230,231] and in Latin America [232], respectively.

Class B β-lactamases: This family includes the enzymes IMP, VIM, SIM, SPM and NDM (Table 4), which are inhibited *in vitro* by ethylenediaminetetraacetic acid (a chelator of Zn^{2+} and other divalent cations) but not by clavulanic acid. These enzymes catalyse the hydrolysis of all β-lactam antibiotics (with exception of aztreonam), conferring high levels of resistance to carbapenems and can be located either in the chromosome or in plasmids. MBL-producing *Acinetobacter* spp. have been reported in clinical and non-clinical niches, mainly in Asian and European countries, including in Portugal (Figures 11 and 13) [229,233,234].

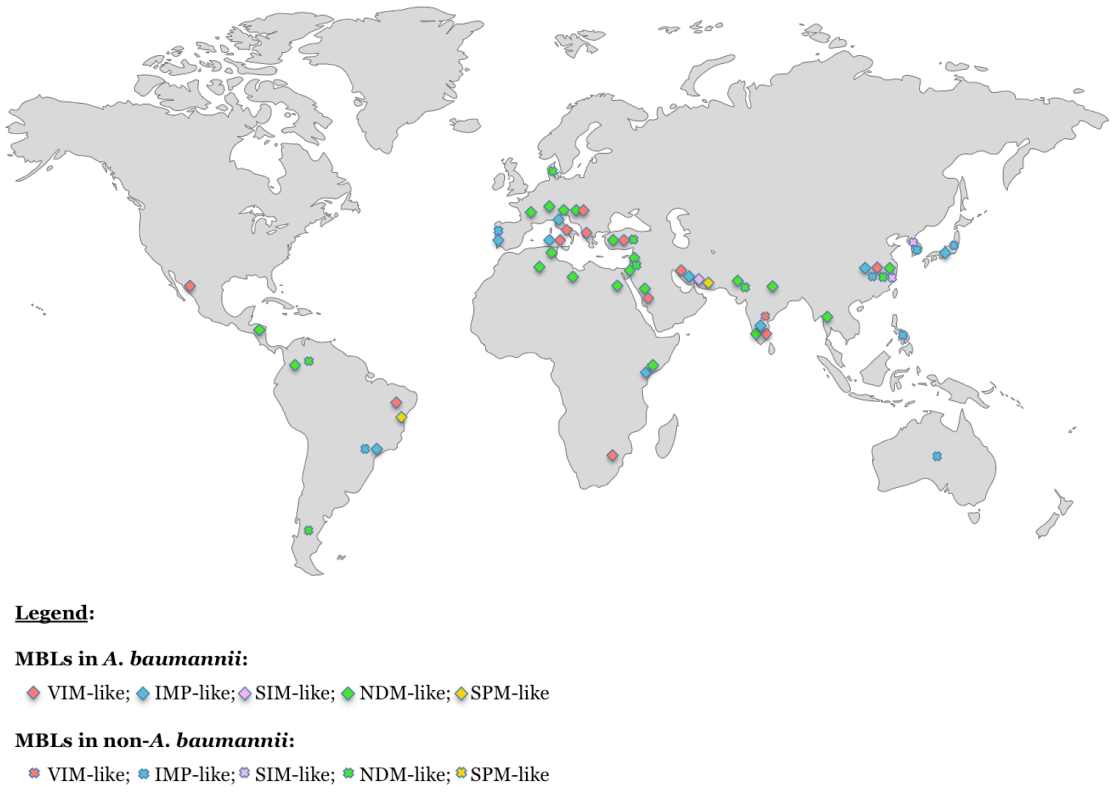


FIGURE 11. Global distribution of carbapenemases (class B) reported in *Acinetobacter* spp. in clinical niches.

Class C β-lactamases: This class of β-lactamases poses therapeutic problems because they can confer resistance to penicillins, cephalosporins, cephamycins and β-lactamase inhibitor combinations, such as clavulanic acid. *A. baumannii* has an intrinsic AmpC cephalosporinase, also called ADC (for ***A**cinetobacter* **d**erived **c**ephalosporinase). Several

allelic variants of this enzyme have been reported in *A. baumannii* with some of them being also found in non-*baumannii Acinetobacter* species (Table 4) (e.g. ADC-5, ADC-12 to -23 in *A. pittii* and ADC-8 in *A. baylyi*) [229,235,236].

Class D β-lactamases: These enzymes are also called oxacillinases - OXAs (in reference to their preferred substrate oxacillin). In *Acinetobacter* species, carbapenem-hydrolysing class D β-lactamases (CHDLs) have been largely identified (including in the environment), being more frequently than class B carbapenemases [237]. Currently, more than 500 different OXAs are known, with many variants possessing carbapenemase activity (https://www.lahey.org/Studies/).

The first acquired CHDL with carbapenemase activity - the **OXA-23** enzyme – was identified in 1985 in Edinburgh, in a multidrug-resistant *A. baumannii* clinical isolate [238]. In the following years, this enzyme and its 26 closely related derivatives (Table 4) have been disseminated worldwide, either in clinical and non-clinical niches (Figures 12 and 13), being the $bla_{\text{OXA-23}}$ gene identified as part of various transposon (Tn) structures, namely Tn*2006*, Tn*2007*, Tn*2008*, Tn*2008B* and Tn*2009*, with plasmid and/or chromosomal location in *A. baumannii* and non-*baumannii* isolates. Interestingly, the natural reservoir of this gene has been identified as *A. radioresistens*, a commensal species which is usually identified on the skin of hospitalized and healthy patients [239,240].

A second group of CHDLs identified on either the chromosome or plasmids in *Acinetobacter* species comprises **OXA-24/40** and its **7** derivatives (Table 4). OXA-24/40 producers were originally recovered in carbapenems-resistant *A. baumannii* isolates in Spain, France, and Portugal, but after that have also been reported worldwide (e.g. USA, Bulgaria, China), including in non-*baumannii* species (e.g. *A. haemolyticus* and *A. calcoaceticus*) (Figures 12 and 13) [241–244]. Although some authors considered that OXA-24/40 show weak activity against cephalosporins and the carbapenems [241], other studies revealed high levels of carbapenems resistance when this enzyme was acquired by a susceptible strain [245].

A third group of CHDLs, represented by **OXA-58** and its 3 variants (Table 4), was first identified in France in 2003 [246]. These enzymes have weak activity against penicillin and carbapenems and ability to hydrolyse cefpirome and cephalothin but not ceftazidime, cefotaxime or cefepime [241]. Like $bla_{\text{OXA-23}}$, epidemiological surveys have identified the $bla_{\text{OXA-58}}$ gene in worldwide isolates of *A. baumannii*, being plasmid-encoded and associated with specific ISs (but are not part of Tns). These ISs play a role in enhancing expression of these enzymes but not in their encoding genes acquisition, which seems to occur by homologous recombination. OXA-58 has also been identified in other

Acinetobacter species, such as *A. junii* [247], *A. pittii* [248], *A. johnsonii* [249] and *Acinetobacter* genomospecies 14TU (Figures 12 and 13) [229].

OXA-143, a fourth group of acquired OXA-type β-lactamases, was discovered in Brazil in 2004. As with other acquired OXA-type β-lactamases, the enzyme kinetics for OXA-143 showed low levels of carbapenems hydrolysis. To date, this group of enzymes (that includes more 4 amino acid variants), has been identified only in *A. baumannii* and *A. pittii* isolates from Brazil, USA, South Korea and Iran and only on plasmids (Figure 12) [250–254].

OXA-235, and the amino acid variants OXA-236, OXA-237 and OXA-278, were identified in *A. baumannii* isolates from the USA and Mexico, and also represent a novel subclass of OXAs with carbapenemase activity (Figure 12) [255].

Besides the acquired CHDLs, *A. baumannii* naturally produces chromosomally-encoded **OXA-51**-group carbapenemases (the largest group of OXA-type β-lactamases identified), some of which could confer resistance to carbapenems when the genetic environment flanking the gene promoted its expression [241] or due to point mutations leading to resistance (e.g. OXA-82) [256]. Other *Acinetobacter* species also present naturally occurring OXAs, such as *A. haemolyticus* (**OXA-214**-like), *A. lwoffii* (**OXA-134**-like), *A. johnsonii* (**OXA-211**-like), *A. radioresistens* (**OXA-23**-like), *A. calcoaceticus* (**OXA-213**-like), *A. bereziniae* (**OXA-229**), and *A. schindleri* (**OXA-235**), which, however, do not confer resistance to carbapenems (Table 4).

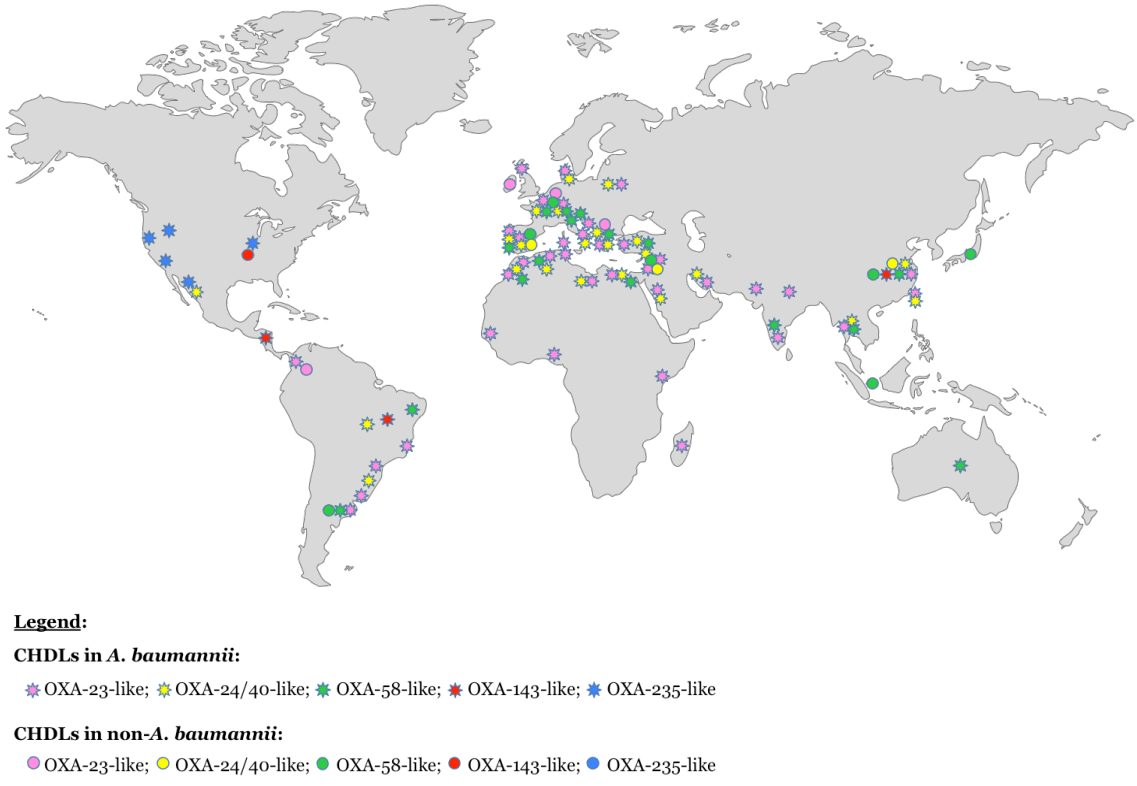


Figure 12. Global distribution of acquired class D carbapenemases reported in *Acinetobacter* spp. in clinical niches.

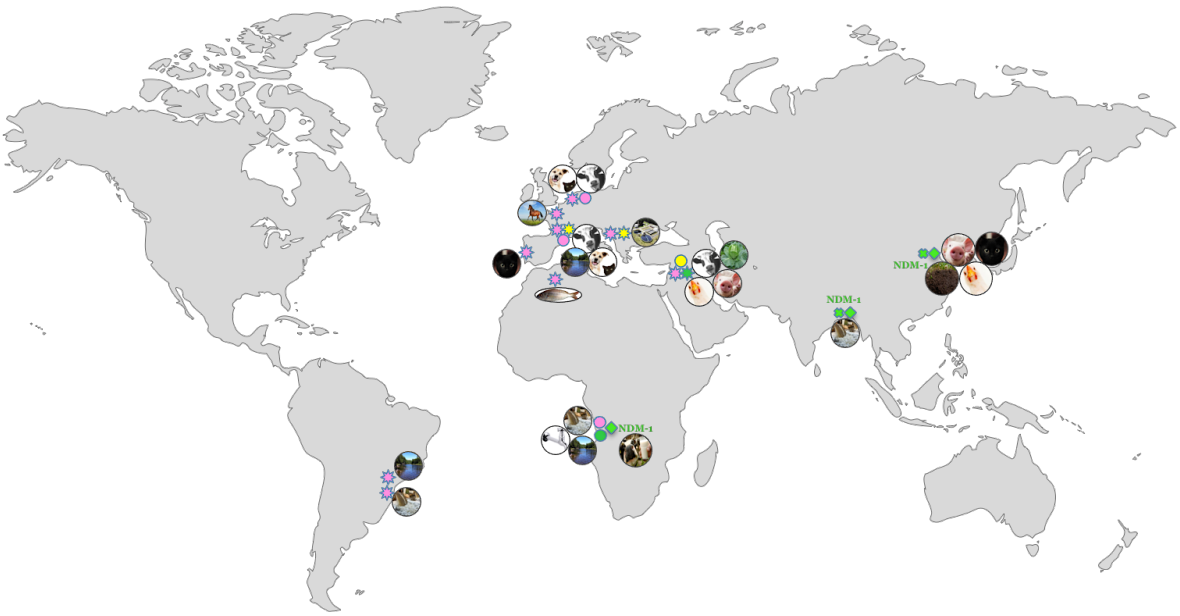


FIGURE 13. Global distribution of acquired carbapenemases (classes B and D) reported in *Acinetobacter* spp. in non-clinical niches [257–265].

❖ Non-Enzymatic mechanisms:

Resistance to β-lactams in *A. baumannii* may be enhanced by the association of β-lactamases with non-enzymatic mechanisms, including alterations on membrane permeability (decrease or absence of porins, overexpression of efflux pumps). Besides the naturally reduced membrane permeability displayed by *A. baumannii* when compared to other Gram-negative organisms (which might be explained by the small number and size of porins), several reports have associated reduced expression of some porins with antimicrobial resistance in this bacterium. These include several Omps, such as the OmpA (also found in other *Acinetobacter* species like *A. radioresistens* and *A. junii*), the Omp22-

33, Omp33-36, Omp37, Omp43, Omp44, Omp47 and CarO (Table 4). Besides outer membrane proteins, envelope components, such as LOS and peptidoglycans, also contribute for antibiotic resistance in *A. baumannii* (e.g. loss or modification of LOS decreases membrane integrity and increases colistin resistance) [168,227].

Antimicrobial efflux is another important mechanism of resistance in *Acinetobacter* spp. In general, efflux constitutes a resistance mechanism that involves the extrusion of antimicrobial agents (as well as other compounds) from the inner side of bacterial membranes to the external environment by means of specific proteins typically named efflux pumps. Although only a few efflux systems have been described in this genus, they contribute to the greatly feared MDR phenotype of nosocomial *Acinetobacter* species [266]. The vast majority of studies regarding efflux in *Acinetobacter* spp. are focused on *A. baumannii* and very little is known about both the presence and the impact of this resistance mechanism in other *Acinetobacter* species [229]. Among the six categories of efflux pumps that have been associated with antimicrobial resistance in *A. baumannii*, the most prevalent belong to the resistance-nodulation-division (**RND**) superfamily: **AdeABC**, **AdeFGH** and the **AdeIJK** efflux pumps (Figure 14) [237]. The expression of each pump is tightly regulated by different mechanisms: AdeABC is controlled by a two-component regulatory system AdeRS, encoded by the genes *adeR* (regulator gene) and *adeS* (sensor kinase), AdeFGH is regulated by the LysR-type transcriptional regulator Ade, and the AdeIJK by the TetR-type transcriptional regulator AdeN. Mutations in these regulatory systems lead to the constitutive overexpression of the corresponding efflux pumps [associated with decreased susceptibility to a wide variety of antimicrobials such as aminoglycosides, tetracyclines, chloramphenicol, and meropenem (Table 4)]. Moreover, the AdeIJK is considered to have a predominant role in the intrinsic low-level resistance phenotype of *A. baumannii* to a broad range of antibiotics, that includes β-lactams, fluoroquinolones, tetracyclines (tigecycline), macrolides (lincosamides), chloramphenicol, antifolates and fusidic acid [267]. Two additional RND efflux systems were described among non-*baumannii* species, AdeDE (lacking the outer membrane protein gene) and AdeXYZ in *A. pittii* [229,266]. It is also important to highlight that these mechanisms may also affect other antibiotic classes.

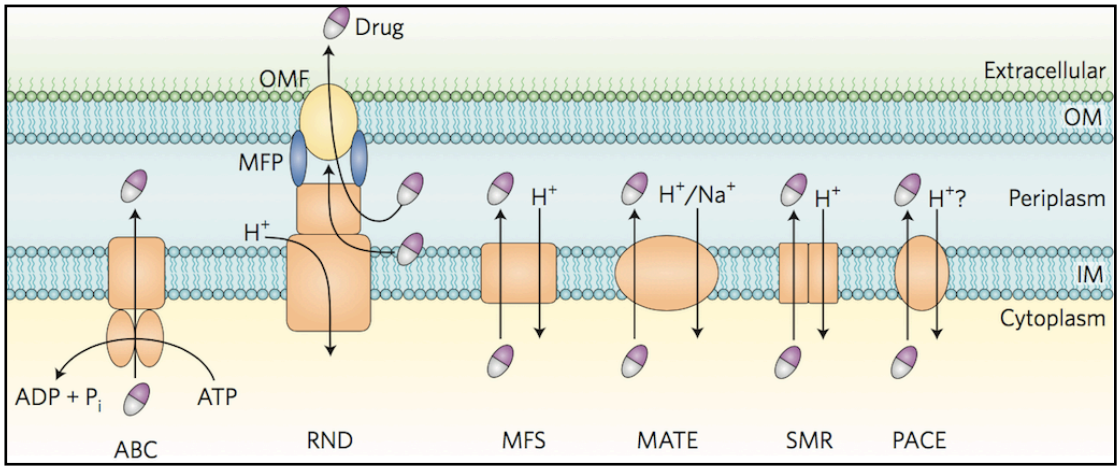


FIGURE 14. Schematic representation of multidrug transporters from the six identified multidrug-resistant superfamilies' in *Acinetobacter* (Table 3). Arrows indicate the pathway of the drug or proton (or ATP)-dependent transport. P_i, inorganic phosphate. Reprinted with permission from [268].

1.1.5.2.2. Resistance to aminoglycosides, tetracyclines, fluoroquinolones and polymyxins

Aminoglycosides are a class of bactericidal antibiotics first developed in the 1940s, that bind to the 16S ribosomal RNA of the 30S ribosomal subunit, inhibiting protein synthesis (Figure 15). These antibiotics can be used parenterally (gentamicin, tobramycin and amikacin), orally (neomycin), by inhalation through a nebulizer (tobramycin) and by intraperitoneal and intraventricular administration (gentamicin) [269]. Despite the nephrotoxicity and ototoxicity associated, this class remains an important alternative therapy for infections caused by MDR strains, usually in combination with other antimicrobials [168]. However, many MDR *A. baumannii* isolates may produce one or more aminoglycoside modifying enzymes (acetyltransferases, phosphotransferases and adenylyltransferases), that lead to aminoglycoside resistance and are usually associated with class 1 integrons harbouring additional antibiotic resistance genes (Table 4). Additionally, a 16S ribosomal RNA methyltransferase ArmA enzyme can confer high levels of resistance to all aminoglycosides [227,270,271]. Moreover, overexpression of efflux pumps such as AdeABC and AbeM (MATE family) also contributes for aminoglycoside resistance (Table 4). This scenario is worsen by the fact that some *A. baumannii* lineages have the ability to develop heteroresistance, a phenomenon in which different bacterial cells within the same population present diverse responses to the antibiotic, thus contributing for the therapeutic failure [272–274].

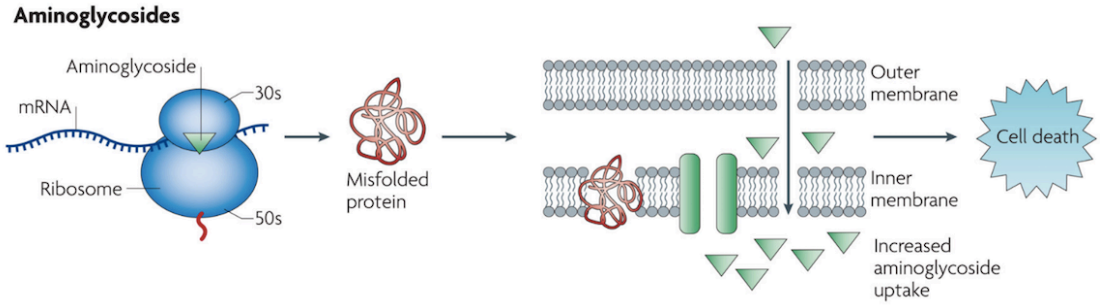


FIGURE 15. Aminoglycosides mechanism of action (reprinted with permission from [226]).

Tetracyclines and glycylcyclines are a broad-spectrum classes of bacteriostatic antibiotics that present a similar mechanism of action [226,275]. They cause conformational changes to the RNA by binding to the 30S ribosomal subunit inhibiting protein synthesis. Tetracyclines class includes tetracycline, doxycycline and minocycline, while glycylcycline includes tigecycline (a semisynthetic derivative of minocycline) [275]. Among Gram-negative organisms, the main mechanisms leading to tetracycline resistance are mediated by active efflux pumps or target protection by production of Tet proteins that bind to the 70S ribosomal subunit. *In vitro*, efflux pumps seen in *A. baumannii* are effective in transporting out tetracycline and doxycycline, with the exception of the TetB pump which is ineffective in transporting out minocycline, remaining this antibiotic (especially if combined with colistin) as a good treatment option for difficult to treat MDR *Acinetobacter* infections, mainly if combined with colistin) [275].

Tigecycline was synthetically designed to overcome these efflux pumps being active in the presence of TetA or TebB. However, rapid development of different resistance mechanisms was observed, being actually associated with the *tetX* gene and, perhaps most importantly for *A. baumannii*, the overexpression of various efflux pumps (AdeABC, AdeIJK, AdeFGH, AbeM, AdeDE) and the upregulation of the macrolide export ATP-binding/permease protein MacB (which is part of the tripartite efflux system MacAB-TolC) [276,277]. Moreover, some studies with *A. baumannii* isolates have revealed that a deletion mutation in the *trm* (which encodes S-adenosyl-L-methionine-dependent methyltransferase), and a frameshift mutation in *plsC* (encoding 1-acyl-*sn*-glycerol-3-phosphate acyltransferase) genes, decreases susceptibility to this antibiotic, although the exact mechanism was not determined [168,278].

Fluoroquinolones are broad-spectrum bactericidal agents (e.g. levofloxacin and ciprofloxacin) that act by binding to bacterial gyrase (encoded by *gyrA* and *gyrB* genes) and topoisomerase IV (encoded by *parA* and *parC* genes) enzymes (Figure 16). Mutations

in the target sites of fluoroquinolones have been extensively reported in *A. baumannii* [278]. In particular, mutations resulting in a Ser-86-Leu substitution in GyrA, and a Ser-80-Leu substitution in ParC increase the MICs of ciprofloxacin in clinical isolates. In *A. baumannii* were also found active efflux by efflux pumps of the RND, SMR and MATE families, responsible for fluoroquinolones resistance (Table 4) [227,279]. All of these mechanisms are chromosomally-encoded and, to date, no plasmid-mediated fluoroquinolones resistance determinants have been identified in *A. baumannii*.

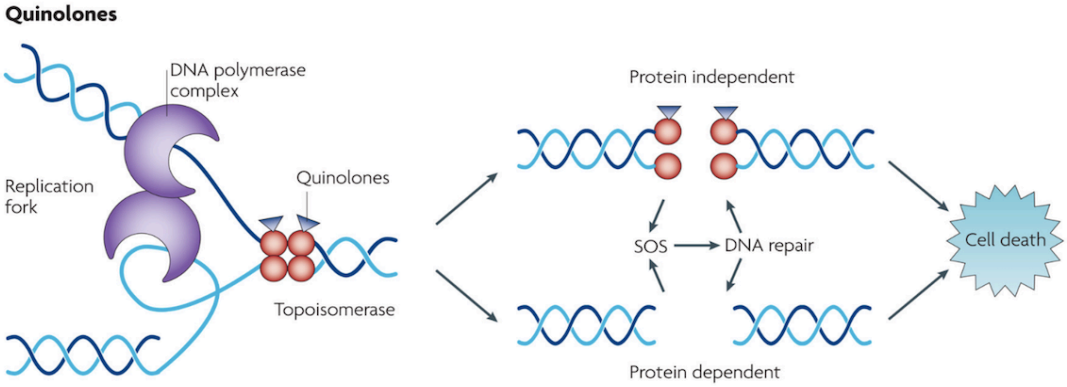


FIGURE 16. Quinolone antibiotics interfere with changes in DNA supercoiling by binding to topoisomerase II or topoisomerase IV (reprinted with permission from [226]).

Polymyxins are lipopeptide bactericidal antibiotics that interact with lipid A, the lipid anchor of LOS of outer membrane of Gram-negative bacteria leading to disruption of membrane, and thereby promoting cell death (Figure 17) [280]. In general, they have a narrow-spectrum, as they are active against common Gram-negative bacteria (e.g. *Acinetobacter* spp., *Klebsiella* spp., *Citrobacter* spp.). These antibiotics, that include colistin (polymyxin E) and polymyxin B, originally discovered in 1940, were abandoned in 1970 due to the high incidence of nephrotoxicity and neurotoxicity, but have been recently re-introduced as last resort drug in the treatment of infections caused by XDR Gram-negative bacteria [281]. Polymyxin resistance remains relatively rare in *A. baumannii* though it can develop after treatment with colistin, in particular in monotherapy. In *A. baumannii*, colistin resistance manifests either through lipid A modifications or by the complete loss of LOS, both of which alter the binding affinity of colistin. Most commonly, colistin-resistant clinical isolates harbour mutations in the two-component regulatory system PmrAB, associated with the addition of phosphoethanolamine (PEtN) by PmrC and galactosamine (GalN) to lipid A, which presumably alters the binding affinity of colistin (Figure 17). The addition of GalN is dependent on the deacetylase activity of NaxD, which is regulated by the PmrAB system [184]. Moreover, the dual activity of the lipid A biosynthesis lauroyltransferase LpxM leads to the constitutive expression of a

predominately hepta-acylated form of LOS, providing increased resistance against colistin. In extreme cases, *A. baumannii* will acquire mutations in the lipid A biosynthetic pathway, thereby halting its production. In the absence of lipid A, *A. baumannii* has been shown to upregulate lipoproteins, namely, A1S_1944, A1S_1945 and A1S_2739, which stabilize the outer membrane. The accumulation of the capsular polysaccharide poly-β-1,6-N-acetylglucosamine (PNAG) has also been observed in strains lacking lipid A as a proposed mechanism of membrane stabilization [184].

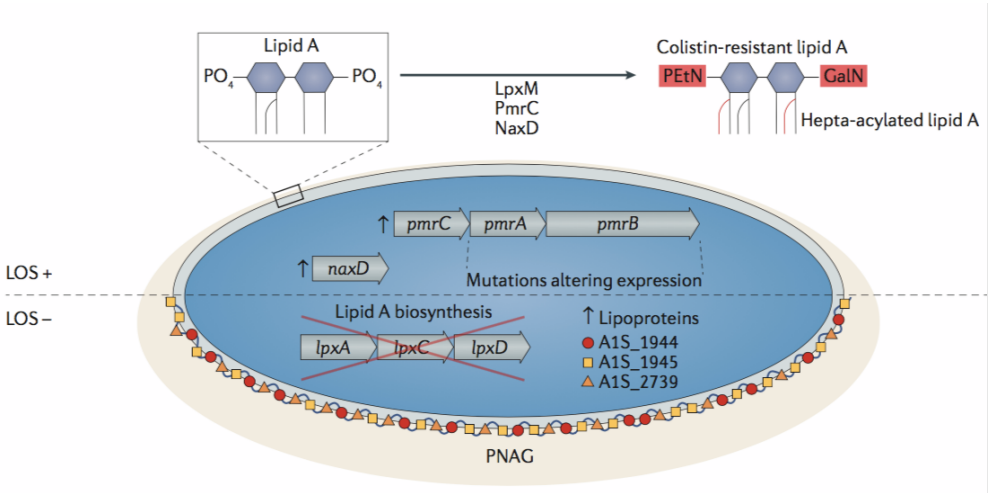


FIGURE 17. Colistin resistance mechanisms of *A. baumannii* (reprinted with permission from [184]). **Abbreviations:** ***lpxA***, acyl-[acyl-carrier-protein]-UDP-N-acetylglucosamine O-acyltransferase, ***lpxC***, UDP-3-O-acyl-N-acetylglucosamine deacetylase, ***lpxD***, UDP-3-O-acylglucosamine N-acyltransferase.

TABLE 4. Major mechanisms of resistance identified in *Acinetobacter* spp. for the different classes of antibiotics. Adapted from [168].

Antimicrobial class/resistance mechanism	Class/family/Type	Protein/variants	References/GenBank accession no.
• **β-lactams/β-lactamases**	**Class A** (Carbenicillinases, Penicillinases, Carbapenemases or ESBLs)	CARB-2, -4, -5, -8, -10 CTX-M-2, -5, -15, -43 PER-1, -2, -7 VEB-1, -1a, -3 TEM-1, -2, -92, -116, -128, -150 SHV-1b, -2, -5, -12, -18, -56, -71, -96 SCO-1 GES-1, -5, -11, -12, -14 KPC-2, -3, -4, -10	[161,167,168,227,237,282,283]
	Class B (MBLs)	IMP-1, -2, -4, -5, -6, -8, -11, -14, -19, -24, -34 NDM-1, -2, -3 SIM-1 SPM-1 VIM-1, -2, -3, -4, -11	[161,168,227,237]; LC276939.1
	Class C (Cephalosporinases)	AmpC (ADC-1 to -8, -10 to -23, -25, -26, -29 to -33, -38, -39, -41 to -44, -50 to -56	[161,167,237]
	Class D (Oxacillinases or CHDLs)		
	• OXA-2-group	OXA-21	[168,227,237,239,241,284]
	• OXA-10-group	OXA-128	
	• OXA-20-group	OXA-37	
	• OXA-23-group	OXA-23, -27, -49, -73, -102, -103, -105, -133, -134, -146, 165 to -171, -225, -239, -366, -398, -422, -423, -435, -440, -481 to -483	
	• OXA-24/40-group	OXA-24/40, -25, -26, -72, -139, -160, -207	
	• OXA-51-group	OXA-51, -64 to -72, -75 to -80, -82 to -84, -86 to -95, -98, -99, -104, -106 to -113, -115 to -117, -120 to -128, -130 to -132, -138, -139, -144, -145, -148 to -150, -160, -172 to -180, -194 to -197, -200 to -203, -206, -208, -216, -217, -219, -223, -241, -242, -248 to -250, -254	
	• OXA-58-group	OXA-58, -96, -97, -164	
	• OXA-134a-group	OXA-134a, -186 to -191	
	• OXA-143-group	OXA-143, -182, - 231, -253, -255	

TABLE 5. *Continued*

Antimicrobial class/resistance mechanism	Class/family/Type	Protein/variants	References/GenBank accession no.
	• OXA-211-group	OXA-211, -212, -309	
	• OXA-213	OXA-213	
	• OXA-214-group	OXA-214, -215	
	• OXA-229-group	OXA-228 to -230, -257	
	• OXA-235-group	OXA-235 to -237, -278	
• OMP alterations		OmpA; Omp22-33; Omp33-36; CarO; Omp37; Omp43; Omp44 and Omp47 kDa proteins	[168,237]
• Efflux pump	RND	AdeABC, AdeIJK	
• Altered PBP expression		PBP2 downregulation	
Tetracyclines			
• Efflux pump	MFS	TetA to TetE	[167,168,237,276,277]
	RND	AdeABC, AdeIJK, AdeFGH	
	ABC	MacB upregulation	
• Ribosomal protection		Tet proteins (e.g. TetM, TetO)	
Glycylcyclines			
• Efflux pump	RND	AdeABC, AdeIJK, AdeFGH	[167,168,237]
Aminoglycosides			
• AME	• Acetyltransferases	AacC1/2, AadA, AadB	[167,168,237]
	• Nucleotidyltransferases	Ant1	
	• Phosphotransferases	AphA1, AphA6	
• 16S rDNA methyltransferases		ArmA	
• Efflux pumps	RND	AdeABC	
	MATE	AdeM	
Quinolones			
• DNA gyrase/topoisomerase mutations		GyrA (Ser-86-Leu substitution); ParC (Ser-80-Leu substitution)	[167,168,237,279]
• Efflux pump	RND	AdeABC, AdeIJK, AdeFGH	
	SMR	AbeS	
	MATE	AbeM	
Chloramphenicol			
	RND	AdeABC, AdeIJK	[167,237]

TABLE 6. *Continued*

Antimicrobial class/resistance mechanism	Class/family/Type	Protein/variants	References/GenBank accession no.
• Efflux pumps	MFS	CmlA, CraA	
	SMR	AbeS	
	MATE	AbeM	
Trimethoprim/sulfamethoxazole			
• Efflux pump	RND	AdeABC, AdeFGH, AbeM	[167,237]
• Dihydropteroate synthase		SulI/II	
• Dihydrofolate reductase		FolA	
Macrolides			
• Efflux pump	MATE, SMR	AbeM, AbeS	[167]
Polymyxins			
PmrAB two-component mutation system		PmrAB (mutations)	[167]
Loss of lipid A of lipopolysaccharide		LpxA, LpxC, LpxD	

Abbreviations: ABC, ATP-binding cassette transporter; **AAC,** aminoglycoside acetyltransferases; **AAD**, Aminoglycoside adenyltransferase; **AbeM,** *A. baumannii* efflux pump of MATE family; **AbeS,** *A. baumannii* efflux pump of SMR family; **Ade,** *A. baumannii* multidrug-resistant efflux pump; **AME,** aminoglycoside-modifying enzymes; **ANT,** aminoglycoside adenyltransferases; **APH,** aminoglycoside phosphotransferases; **ArmA,** armillaria mellea; **CarO,** carbapenem-associated outer membrane protein; **CmlA,** chloramphenicol resistance *Acinetobacter*; **CraA,** chloramphenicol resistance *Acinetobacter* pump; **FolA,** folate; **GyrA/ParC,** DNA Gyrase/partitioning of the nucleoid partition; **MacB,** macrolide export ATP-binding/permease protein; **MATE,** multidrug and toxic compound extrusion efflux pump; **MFS,** major facilitator superfamily; **OMP,** outer membrane protein; **PBP,** penicillin-binding protein; **RND,** resistance-nodulation-cell division; **SMR,** small multidrug-resistant efflux pump.

1.1.5.2.3. Acquisition and dissemination of antimicrobial resistance

1.1.5.2.3.1. Genetic support and expression of antimicrobial resistance genes

The high genetic plasticity presented by *Acinetobacter* and particularly in *A. baumannii*, contributed for the accumulation of many resistance determinants, which resulted in the emergence of MDR strains. One plausible explanation for this capacity is the reduction, or even complete loss of the so called CRISPR-Cas systems. These systems, which are similar to an adaptive immune system, can protect bacteria from foreign genetic elements such as plasmids and bacteriophage phages infection and thus, their loss would enable bacteria to acquire genetic elements more easily [285]. In fact, it is proposed, not only for *Acinetobacter*, but also for other critical bacterial pathogens such as *E. faecalis* and *K. pneumoniae* inverse correlations between antibiotic resistance and the presence of CRISPR-Cas loci [286].

Acinetobacter genomes display a wide variety of mobilizable and mobile genetic elements (MGEs) carrying antibiotic resistance genes, such as integrons, transposons, which may also be included in genomic islands [287–289], as well as in plasmids. Besides mobilization, these elements assure often the expression level of the associated genes. In this section, an overview of the main MGEs linked to carriage of *bla* genes and multidrug resistance phenotypes in *Acinetobacter* species will be presented (table 5). For concepts regarding mobile and mobilizable genetic elements (see Box 7).

Integrons: Class 1 integrons are the most widespread and clinically relevant in *Acinetobacter* sp. isolates, although other classes may also occur (e.g. class 2 integrons in Latin American countries) [290–292]. The dissemination of integrons occurs through association with transposons: class 1 integrons are usually associated with functional or non-functional transposons derived from Tn*402* which may be inserted into larger transposons such as Tn*21*; whereas class 2 integrons are usually associated with Tn*7* family [293–295]. More than 130 different gene cassettes encoding antibiotic resistance have been identified in integrons. Table 5 summarizes the main integrons structures associated with β-lactamase genes in *Acinetobacter* species.

Insertion Sequences: Bacterial insertion sequences (ISs) are the simplest type of transposable elements and very frequent among *Acinetobacter* (from 0 to ~400 per genome). In addition, when two copies of the same IS are flanking a resistance gene they form a complex structure called composite or class 1 transposon, which can mobilize a

variety of resistance genes, contributing to antimicrobial resistance dissemination [293]. ISs also contribute to increase the expression of neighbour genes due to the presence of promotor regions. IS*Aba1*, IS*Aba2*, IS*Aba3*, IS*Aba4*, IS*Aba10*, IS*Aba125*, and IS*18* are the most frequent ISs associated with the expression of CHDL genes in *A. baumannii* (Table 5). IS*Aba1* (IS4 family) has been found upstream the $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-58-like}}$ and bla_{AmpC} genes in *A. baumannii*, being proposed that is "customized" for *Acinetobacter* species, since it contains promoter sequences recognized by *Acinetobacter* typical transcription factors [296–298]. In some cases, an IS can be disrupted by other (or more) ISs, which provides additional promoter sequences for the resistance genes [299]. Besides being involved in the expression and spread of CHDLs, some ISs have also been associated with MBLs (e.g. IS*Aba125*) and class A β-lactamases (e.g. IS*Ecp1*), and can be associated with reduced susceptibility to carbapenems by disruption coding sequences of certain genes (e.g. *carO* gene) [300,301].

Transposons: In *A. baumannii*, transposons have been characterized as genetic structures harbouring important resistance genes, such as $bla_{\text{OXA-23}}$. Five transposons have been related to this carbapenemase gene (Tn*2006*, Tn*2007*, Tn*2008*, Tn*2008b* and Tn*2009*), all of them globally disseminated except Tn*2009*, identified only in China (Figure 18) [239,293,297,302]. Despite all of these structures have been named as transposons, only Tn*2006* and Tn*2009* are typical class 1 transposons, with two copies of IS*Aba1* in opposite or the same orientation, respectively, flanking an internal segment containing $bla_{\text{OXA-23}}$. Tn*2008* and Tn*2008b* are similar to Tn*2006* but the first two lack one copy of IS*Aba1* and in the second the two IS*Aba1* elements were transcribed in the same orientation. Tn*2007* is associated with one copy of IS*Aba4*, which is located upstream the $bla_{\text{OXA-23}}$ gene, but there is currently no evidence that this transposon is mobilizable [239].

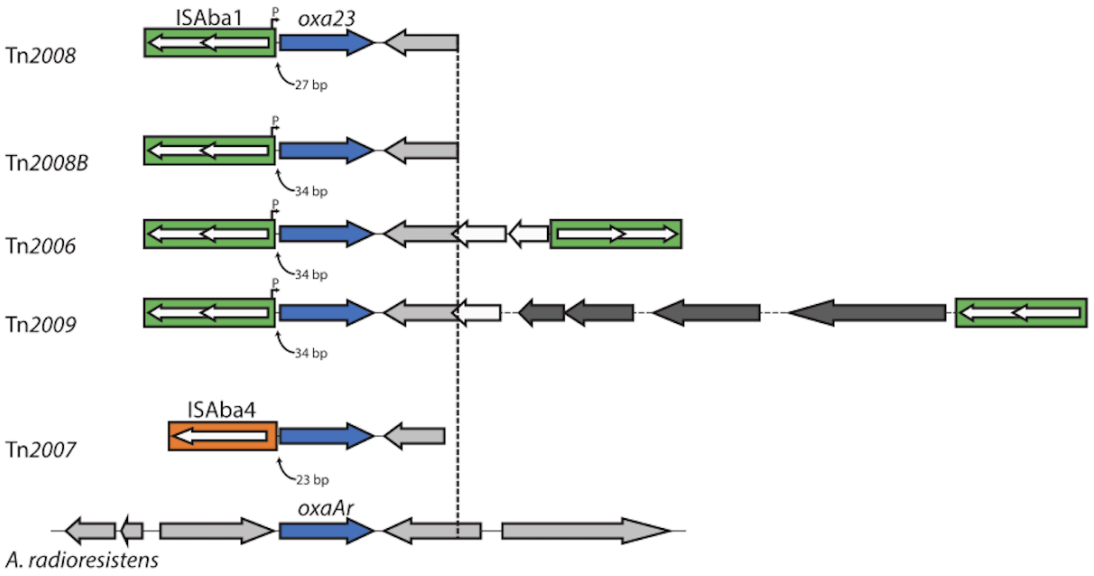


FIGURE 18. The five known structures harbouring bla_{OXA-23} in *Acinetobacter*. Reprinted with permission from [239].

Genomic Islands (GI): Genomic islands were discovered for the first time in *A. baumannii* by Fournier and colleagues [287]. The first reported, AbaR, the first island described, comprises a transposition module (*orf1*, *tniA*, *tniB*, *orf2*, *orf3*) and two other genes encoding an universal stress protein (*uspA*) and a sulphate permease (*sul*) [287,303,304]. Since then, several others GIs were described, mainly among *A. baumannii* but also in other *Acinetobacter* species, and particularly in epidemic lineages [305]. These islands, usually contain a variety of genes encoding resistance to multiple antibiotics and heavy metals, and most of them are preferentially incorporated into a specific location within the *comM* gene [306,307]. The bla_{OXA-23} gene is one of the resistance genes found associated with an antibiotic resistance island, Tn*6167*, in an ST208^{Ox} isolate from Australia [91]. The large majority of GIs was found in strains belonging to IC I (e.g. AbaR1, AbaR3, AbaR5, AbaR6, AbaR7, AbaR8, AbaR9, and AbaR10). All except the AbaR6 and AbaR7, share a common structure represented by a backbone transposon (Tn*6019*), interrupted by a large compound transposon that contains a variable-resistance region bounded by directly oriented copies of Tn*6018*. Conversely, much less is known about the resistance islands harboured by IC II this clone [308].

The largest resistance island described to date is AbaR1 and was described in IC I epidemic *A. baumannii* lineage AYE strain. This island contains an 86 kb region with a G+C content of 52.8% (in opposition to the typical 38.8% of *A. baumanni* chromosome), and many resistance determinants (including genes that had not been previously

described in *Acinetobacter* species) [287]. AbaR2, in turn, was described in IC II epidemic and MDR *A. baumannii* strain ACICU, a strain that carries the plasmid-mediated bla_{OXA-58} [287,309]. Finally, AbaR3 contains eight genes associated with antibiotic resistance (e.g. bla_{TEM} gene associated with a Tn*3* transposon) [310,311].

Plasmids: *Acinetobacter* plasmids range from 2 kb to more than 100 kb in size [84,312–315]. The large plasmids of *A. baumannii* are characterized by the presence of multiple antibiotic resistance genes and are usually self-transmissible [314,316–318]. Despite the importance of plasmids in the potential transmission of antibiotic resistance and virulence genes in *A. baumannii*, we know very little is known regarding the basic replicons of these plasmids, their replication mechanisms and transmissibility. So far, *Acinetobacter* plasmids have been classified according to the replicase (Rep) proteins [plasmid-based replicon typing scheme (PBRT), being actually known 19 homology groups (GR1-GR19)] [105]. The majority of plasmids from *Acinetobacter* encode replicase proteins belonging to the Rep-3 superfamily with the larger plasmids usually harbouring more than one replicon type [105]. CHDLs genes were found to be particularly associated with rep*Aci6* and rep*Aci1* (bla_{OXA-23}), rep*Aci1*, rep*Aci3*, rep*Aci4,* rep*Aci6*, rep*Aci9*, rep*Aci10* and rep*AciX* (bla_{OXA-58}), and rep*Aci2*, rep*Aci6*, p2ABSDF0001, and repA_AB (bla_{OXA-40}) [245,315,319]

BOX 7

Glossary - Mobile and mobilizable genetic elements concepts

Integrons - genetic units able to capture, mobilize and express genes that are contained in mobile elements called gene cassettes. Integrons are organized in three regions: the 5´conserved region, the 3´conserved region, and a variable region. The 5´region consists of the ***int*** gene, encoding a site-specific recombinase of the integrase family, an ***att*** adjacent site recognized by the integrase and acting as a receptor site for gene cassettes, and a **Pc promoter** for gene cassette expression [320]. They are not mobile by themselves (but gene cassettes can readily be exchanged to other integrons), but they can spread to different chromosomal locations or plasmids by insertion sequences (IS)-mediated transposition or homologous recombination. According to the integrase sequence (*int*) they can be classified into **five different classes**. In class 1 integrons, the 3´conserved region often consists of a partially deleted *qac* gene (*qacEΔ1*) fused to a *sul* gene conferring resistance to quaternary ammonium compounds and sulphonamides, respectively.

Gene cassettes - small mobilizable elements comprising **one gene** or open reading frame (often an antibiotic resistance gene) and a recombination site (***attC***), known as 59-be element, that allows them to be recognized by the integrase (*intI*) [294].

Transposable elements - comprise IS and transposons [293].

Insertion sequences (IS) - correspond to the least complex transposable element, consisting on a gene codifying for a transposase, usually flanked by inverted repeats (IR) sequences (single

stranded sequence of nucleotides followed downstream by its reverse complement). IS are able to move from one location to another in the genome (causing insertion mutations and genome rearrangements), and they can exist as autonomous or as part of class 1 transposons [321,322]. Besides their transposition role, some IS have been shown to activate or to increase the expression of neighbour genes [293].

Transposons - these elements may vary in size from 3 to 40 kb and are divided into two main classes: **composite** (class I) or **complex** (class II) transposons. Composite transposons have antibiotic resistance genes in its central region, flanked by the same IS (or two closely related) at each end. Complex transposons include genes coding for a transposase (*tnpA*, enzyme involved in excision and integration) and resolvase (*tnpR*, a site-specific recombinase for co-integrate resolution within the *res* site), and one variable DNA fragment flanked by two IR sequences [293,295].

Genomic islands (GIs) - large clusters of genes involved in horizontal gene transfer that can be mobilized by, for example, plasmids or phages. GIs are flanked by direct perfect repeats and usually carry mobility genes coding for integrases or transposases that are needed for chromosomal integration and excision, and also other additional genes codifying for adaptive features. GIs are usually identified due to differences in G+C content from the rest of the genome and by the presence of direct repeats at their ends.

Plasmids - extra chromosomal genetic units able to replicate autonomously in a given host bacterial cell, and which can be transmitted to other cells by conjugation, transformation or transduction. Plasmids comprise genes coding for essential (replication, maintenance and transfer) and adaptive (e.g. antibiotic resistance genes, metal tolerance, virulence) functions and may cross many species and genus barriers, contributing for a successful dissemination [323]. They can be classified according to the number of copies, host range, replication (incompatibility group) and mobility. According to the **mobility**, plasmids can be classified as **conjugative** or **mobilizable**. The mobility machinery includes a set of plasmid mobility (MOB) genes [*oriT*, relaxase and type IV coupling proteins (T4CP)] necessary for the conjugative process, and a mating pair formation (MPF) complex which is a type 4 secretion system (T4SS) [324–326]. By definition two plasmids that belong to the same Inc group cannot stably coexist in the same cell. Mobilizable plasmids (Figure 19) use the T4SS of another genetic element present in the cell to be transferred, are usually smaller and present in high copy numbers [324]. Conjugative plasmids encode the functions needed for their own transfer (MOB and MPF), replication and stability, and are generally large (>30 kb) and appear in low copy numbers in the bacterial cell.

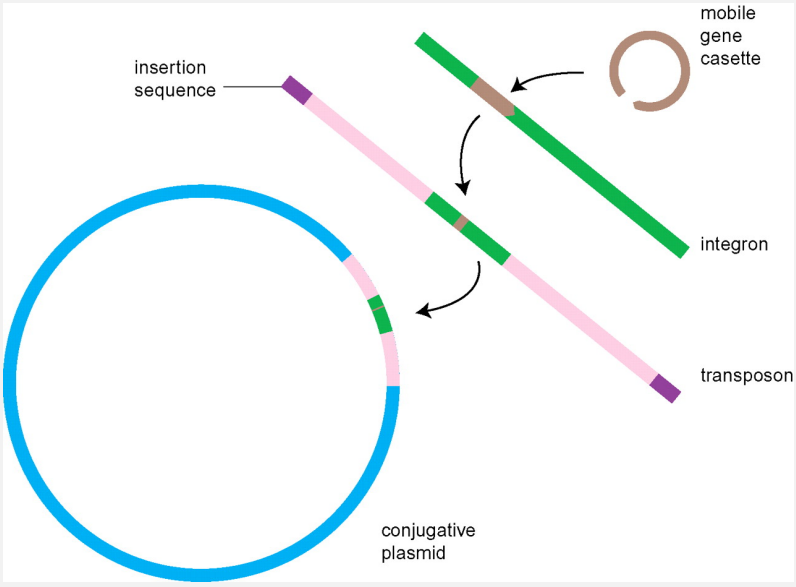


FIGURE 19. The modular and hierarchical composition of MGEs. Reprinted with permission from [324].

TABLE 7. Characterization of the main MGEs associated with the most widespread *bla* genes in *Acinetobacter* spp. Adapted with permission from [161].

Antibiotic resistance gene		Genetic platform	*Acinetobacter* spp.	GenBank accession no. & References
Class B	***bla*$_{IMP-1}$**	*intI1-bla*$_{IMP-1}$*-aac6-II-aadA4-dfrA1-orfC* ***In86****: intI1-bla*$_{IMP-1}$*-aac(6´)-31-aadA1-qacEΔ1* *intI1-aacA4-bla*$_{IMP-1}$*-bla*$_{OXA-2}$*-qacEΔ1*	*A. pittii* *A. baumannii* *A. baumannii, A. bereziniae*	[327] [328] EF375699.1, EU686386.1
	***bla*$_{IMP-2}$**	*intI1-bla*$_{IMP-2}$*-aacA4-aadA1*	*A. baumannii*	[329]
	***bla*$_{IMP-4}$**	*intI1-bla*$_{IMP-4}$*-qacG2-aacA4-catB3*	*A. baumannii*	AF445082.1
	***bla*$_{IMP-5}$**	**In76 (Tn402-like):** MITE-*intI1-bla*$_{IMP-5}$*-qacEΔ1-sul1-orf5-tniBΔ1-tniA*-MITE	*A. baumannii*	[234]
	***bla*$_{IMP-8}$**	*intI1-bla*$_{IMP-8}$*-aac(6´)-Ib* *intI1-bla*$_{IMP-8}$*-aac(6´)II-aadA4-qacEΔ1*	*A. baumannii* *A.baumannii, A. pittii*	DQ845788.1 [327]
	***bla*$_{IMP-19}$**	**In477:** *intI1-bla*$_{IMP-19}$*-aac(6´)-31-bla*$_{OXA-21}$*-aadA1*	*A. baumannii, A. bereziniae, Acinetobacter genomosp. 16, A. guillouiae, A. junii, A. nosocomialis, A. pittii*	[330,331]
	***bla*$_{IMP-34}$**	*intI1-bla*$_{IMP-34}$*-aacA4-bla*$_{OXA-1}$*- qacEΔ1-sul1-NAT-tniBΔ*	*Acinetobacter genomosp. 13BJ*	LC276939.1
	***bla*$_{VIM-1}$**	*intI1-bla*$_{VIM-1}$*-qacEΔ1* *intI1-orf11-aacA(6´)-Ib-bla*$_{VIM-1}$*-qacEΔ1* *intI1-bla*$_{VIM-1}$*-aacA7-dhfrI-aadA1-qacEΔ1-sul1*	*A. baumannii* *A. baumannii* *A. baumannii*	EF690696.1 EF690695.1 [332]
	***bla*$_{VIM-2}$**	*intI1-bla*$_{VIM-2}$*-aacA7-aadA1-qacEΔ1* *intI1-bla*$_{VIM-2}$*-aacA4-aadA1-orfII-orfIII-qacEΔ1* *intI1-bla*$_{VIM-2}$*-aadB-ISpa21-like-bla*$_{OXA-2}$ *intI1-aacA7-bla*$_{VIM-2}$*-aacA1-qacEΔ1-sul1-orf-tniBΔ1-tniA*	*A. baumannii, A. bereziniae* *A. pittii* *A. baumannii* *A. bereziniae*	AF324464.1, EU014075.1 AF369871.1 AM749812.1 JX235356.1
	***bla*$_{VIM-3}$**	*intI1-bla*$_{VIM-3}$*-orf2-aacA4-qacEΔ1*	*A. baumannii*	[333]
	***bla*$_{VIM-11}$**	*intI1-bla*$_{VIM-11}$	*A. baumannii*	EF116550.1
	***bla*$_{SIM-1}$**	*intI1-bla*$_{SIM-1}$*-arr3-catB3-aadA1-qacEΔ1*	*A. baumannii*	[334]
	***bla*$_{NDM-1}$**	**Tn*125*:** IS*Aba125-bla*$_{NDM-1}$-IS*Aba125* **Tn*125*:** IS*Aba125-bla*$_{NDM-1}$*-ble-trpF-tat-dct-groES-groL*-IS*91*-IS*Aba125* **Tn*125*:** IS*Aba125-bla*$_{NDM-1}$*-ble-Δiso-tat-dvt-groES-groEL-ISCR21-Δpac*-IS*Aba125* **Tn*125*:** IS*Aba125-bla*$_{NDM-1}$*-ble-trpF-dsbC-cutA1-groES-groEL-insE-pac*-IS*Aba125* **Tn*125*:** IS*Aba125-bla*$_{NDM-1}$*-ble-trpF-tat-cutA1-groES-groEL-insE-Δpac*-IS*Aba125*	*A. baumannii* *A. towneri* *A. baumannii* *A. bereziniae* *A. pittii*	[335] KT965093.1 JN872329.1 KT072713.1 KR153289.1
	***bla*$_{NDM-2}$**	**Tn*125*:** IS*Aba125-bla*$_{NDM-2}$*-ble-ΔtrpF-*IS*Aba125* **Tn*125*:** IS*Aba125-bla*$_{NDM-2}$*-ble-Δiso-tat-dvt-groES-groEL*-IS*CR21-Δpac*-IS*Aba125*	*A. baumannii* *A. baumannii*	[336] [337]
Class D	***bla*$_{OXA-2-like}$**	*intI1-aacA4-bla*$_{IMP-1}$*-bla*$_{OXA-2}$*-qacEΔ1* *intI1-bla*$_{VIM-2}$*-aadB*-IS*pa21-like-bla*$_{OXA-2}$ *intI1-aadB-bla*$_{OXA-21}$ **In477:** *intI1-bla*$_{IMP-19}$*-aac(6´)-31-bla*$_{OXA-21}$*-aadA1*	*A. baumannii, A. bereziniae* *A. baumannii* *A. baumannii* *A. baumannii, A.*	EF375699.1, EU686386.1 AM749812.1 [338] [330,331]

Antibiotic resistance gene		Genetic platform	*Acinetobacter* spp.	GenBank accession no. & References
			bereziniae, Acinetobacter genomosp. 16, A. guillouiae, A. junii, A. nosocomialis, A. pittii	
	***bla*OXA-10-like**	*intI1*-IS*1999*-*bla*VEB-1-*aadB-arr2-cmlA5-bla*OXA-10-*aadA1-qacEΔ1-sul1* *intI1-aadB-cat-bla*OXA-10-*aadA1* *intI1-bla*OXA-10-*aacA4-bla*IMP-55-*cmlA5* **In3-6:** *intI3-aac(6´)Ib-bla*OXA-10-*aadA1-like-qacE2-like*	*A. baumannii* *A. baumannii* *A. baumannii* *A. johnsonii*	[161] DQ288250.1 KT935306.2 [320]
	***bla*OXA-20-like**	*intI1-aacA4-orfO-bla*OXA-20-*qacEΔ1* *intI1-aacA4-bla*OXA-37	*A. baumannii* *A. baumannii*	[339,340] [341]
	***bla*OXA-23-like**	**Tn*2006*:** IS*Aba1-bla*OXA-23-*ATPase*----IS*Aba1* **Tn*2007*:** IS*Aba4-bla*OXA-23-*ATPase* **Tn*2008*:** IS*Aba1-bla*OXA-23-*ATPase* **Tn*2008b*:** IS*Aba1-like-bla*OXA-23-*ATPase-like* **Tn*2009*:** IS*Aba1-bla*OXA-23-*ATPase-orf1-orf2-orf3-orf4*-IS*Aba1*	*A. baumannii, A. nosocomialis* *A. baumannii* *A. baumannii, A. baylyi, A. radioresistens* *A. baumannii, A. radioresistens* *A. baumannii, A. junii*	[299] [298]; KP888560.1 [298]; JF731029.1 [239] [239,302,342,343]
	***bla*OXA-51-like**	IS*Aba1-bla*OXA-51-like IS*Aba9-bla*OXA-51 **Tn*6080*:** IS*Aba1-bla*OXA-51-like*-str-orf14*	*A. baumannii* *A. baumannii* *A. baumannii*	[344–347] [344] [348]
	***bla*OXA-58-like**	IS*Aba3-like*-IS*Aba825*-IS*Aba3-like-bla*OXA-58-IS*Aba3* IS*Aba125*-IS*Aba3-like-bla*OXA-58-IS*Aba3-araC1-lysE*-IS*Aba3-like*-IS*Aba125*-IS*Aba3-like-bla*OXA-58 IS*Aba3-like*-IS*Aba2*-IS*Aba3-like-bla*OXA-58-IS*Aba3* IS*26*-IS*Aba2-bla*OXA-58-IS*Aba3*-IS*26* IS*Aba2-bla*OXA-58-IS*Aba3*-IS*26*-IS*Aba2-bla*OXA-58-IS*Aba3*-IS*26* IS*Aba2-bla*OXA-58-IS*Aba3* IS*18-bla*OXA-58-IS*Aba3* IS*Aba1-bla*OXA-58-IS*Aba3* IS*Aba3-bla*OXA-58-IS*Aba3* *bla*OXA-97-IS*Aba3*-araC1-*lysE*-araC2-*reg-orf*-IS*Aba8* IS*Aba3-like*-IS*1006-bla*OXA-58-IS*Aba3* IS*1008*-ΔIS*Aba3-bla*OXA-58 IS*Aba2*-ΔIS*Aba3-bla*OXA-58-IS*Aba3* IS*18*-ΔIS*Aba3-bla*OXA-58-IS*Aba3*	*A. baumannii* *A. pittii* *A. baumannii* *A. baumannii* *A. baumannii* *A. baumannii* *A. baumannii* *A. pittii* *A. baumannii Genomosp. 13TU* *A. baumannii* *A. baumannii* *A. baumannii*	[349] [350] [297] [351] [351] [352] [352] [352] [327] [353] [354] [355] [297] [297]
	***bla*OXA-143-like**	XerC/XerD-*bla*OXA-253 *rep-mob-bla*OXA-143-*rep* **pAb25:** *tonB-orf2-orf1-bla*OXA-253-*repA-repB*	*A. baumannii* *A. baumannii* *A. baumannii*	KC479324.2 GQ861437.1 KF824909.1

1.1.5.2.3.2. Population structure of carbapenem-resistant *A. baumannii*

The global population structure of multidrug-resistant and carbapenem non-susceptible *A. baumannii* isolates is characterized by a low diversity of clones/clonal complexes (CCs). Initial typing studies attributed the spread of MDR *A. baumannii* to three predominant clonal lineages, initially designated as European clones I-III [96,356]. However, these three European clones have subsequently become endemic in many worldwide countries, being accordingly re-named as "international clones (ICs) I–III" [83], whose diversity was uncovered with the use of MLST. CC109^{Ox}/CC1^{Pa}, CC92^{Ox}/CC2^{Pa} and CC187^{Ox}/CC3^{Pa}, representing international clones I, II and III respectively; where "Ox" refers to Oxford MLST scheme and "Pa" to Institute Pasteur MLST scheme. They are globally disseminated, including in non-clinical niches (e.g. in animals), which is suggestive of their adaptation to diverse habitats [357]. Among the numerous STs included in these two CCs, ST92^{Ox} and ST109^{Ox} (which were further reassigned as ST208 and ST231, respectively) [358] are the most disseminated, being frequently associated with genes encoding resistance to carbapenems (e.g. *bla*$_{VIM\text{-}like}$, *bla*$_{OXA\text{-}23\text{-}like}$, *bla*$_{OXA\text{-}24\text{-}like}$, and/or *bla*$_{OXA\text{-}58\text{-}like}$) or aminoglycosides (*armA*, a 16S rRNA methylase) [359]. Worryingly, the occurrence of ST92^{Ox}/ST208^{Ox} isolates resistant to all available antimicrobial agents has already been described [84,360].

Actually, almost 1900 MLST sequence types (STs) are listed in the *A. baumannii* MLST^{Ox} database and some other highly successful lineages are known to be widely distributed [83,94,352,361,362]. Some of these "new" epidemic lineages (e.g. CC103^{Ox}/CC15^{Pa}, CC110^{Ox}/CC25^{Pa}, CC113^{Ox}/CC79^{Pa}) in some cases, harbour subtypes with distinctive temporal and geographical distributions [83,363,364].

In Portugal, particular clones have dominated over the last years: the ST98 producing OXA-40 was dominant until 2006 and subsequently replaced by the worldwide epidemic ST208 producing OXA-23. The specific determinants favouring transmission, colonization, and/or invasion, and hence the successful spread and epidemic behaviour of these clones, still have not yet be completely defined [84]. However, their remarkable capabilities for antibiotic resistance determinants and mobile genetic elements acquisition, as well as for biofilm formation, together with the inter-hospital transfer of colonized patients, seem to be also important factors that need to be further clarified [365].

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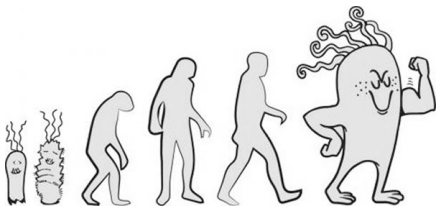


Chapter 2

Objectives and outline of the study

"Imagination is more important than knowledge."

Albert Einstein

2.1. Statement of objectives

In the last decades, we have witnessed a dramatic increase in antimicrobial resistance of infectious bacterial agents, representing a difficult challenge for healthcare institutions all over the world. *Acinetobacter* species are among the most concerning bacteria, showing a prodigious ability to become resistant to multiple antimicrobial agents (including carbapenems), and to persist in the hospital environment. Although *A. baumannii* is still considered the most prevalent *Acinetobacter* species within the hospital setting, the number of reports of infections caused by non-*baumannii* species and associated with multidrug resistant strains increased in the last years.

A lasting (1995-2008) high occurrence of carbapenem-resistant *A. baumannii* in Portuguese hospitals was previously associated with an interesting clonal dynamic, supported by the expansion of specific CHDL-harbouring clones in different time-periods (2002-2006 ST98-OXA-24/40; 2001-2004 ST103-OXA-58; 2006-2010 ST208-OXA-23). The enlargement on the antibiotic resistance profile was associated with this clonal switch. Nevertheless, other clonal features (e.g. resistance to other antimicrobial agents, virulence traits or resilience traits) as well as the relevance of other settings for the emergence and proliferation of particular *A. baumannii* clones are still poorly understood. Moreover, and although less explored, the interspecies transfer of resistance traits that occur between non-*baumannii* and *A. baumannii* species (e.g. the previously reported $bla_{\text{OXA-40}}$ associated plasmids), also account for carbapenem resistance, with non-*baumannii* species/non-hospital niches potentially constituting an important source/reservoir of relevant resistance genes that is worth to be explored.

The increasing use of DNA-based strain typing tools like MLST and WGS is contributing for a more precise definition of the population structure and antibiotic resistance drivers among *Acinetobacter*. However, most of these methods are still too expensive, time-consuming and/or require specialized skills, hindering their implementation in routine clinical laboratories. Thus, alternative methods offering a high-throughput, low-cost and quick performance are highly required in order to block transmission and support appropriate therapeutic decisions. Some progress have been attained with FTIR spectroscopy for some bacterial species, or MALDI-TOF MS spectrometry, an already thoroughly adopted technique in clinical microbiology laboratories for species identification, paving the way for further developments on *Acinetobacter* and envisioning a bright future for real-time application for typing. Since these methodologies are based on different non-DNA-based principles and target different biomolecules from the bacterial cell, they provide an opportunity to integrate multidisciplinary data (genomics, proteomics, metabolomics), and can additionally

provide relevant insights into key molecular features that can be of utmost interest to understand bacterial evolution, pathogenesis and host-pathogen interactions.

The **main goals of this Thesis** were:

- To identify the main features contributing for the clonal dynamics and success of particular *A. baumannii* lineages and also to understand the role of *non-baumannii Acinetobacter* species in the dissemination of carbapenem resistance.
- To explore the potential of different DNA- and non-DNA-based high-throughput omic technologies (WGS, FTIR and MALDI-TOF MS) for *Acinetobacter* characterization and accurate typing, at species and sub-species level.

To accomplish these purposes, a recent Portuguese collection of *Acinetobacter* spp. clinical isolates (2010-2015), selected based on the origin (nosocomial and community infections, type of infection, different wards), geographical region (North and Centre) and antibiotic susceptibility profile, was screened for bacterial species, clones, and genomic pieces contributing to virulence and adaptation to different stressors, with emphasis to carbapenems. Furthermore, the occurrence, diversity and antimicrobial resistance of a collection of *Acinetobacter* species from non-clinical sources from Benguela, Angola (healthy volunteers, aquatic environments, healthy animals and their environments; 2013) was also evaluated, as part of a larger antimicrobial resistance surveillance project in this region. Finally, the potential of FTIR and MALDI-TOF MS for *Acinetobacter* species identification and typing was also explored.

The **specific aims** of this thesis were:

1. To characterize the phenotypic and molecular features of recent *A. baumannii* clinical isolates [two hospitals, one Long-Term Care Facility and one Community Clinical Laboratory (2010-2015)] in order to identify the clones currently causing infection in our area and identify potential important reservoirs and transmission routes. Comparison of this data with the ones from previous collections will help on the identification of key features contributing for the success of particular lineages.

2. To characterize the carbapenem resistance mechanisms and mobile genetic elements responsible for the spread of carbapenem resistance determinants among

different *Acinetobacter* species.

3. To assess the pathogenicity potential and the possibility of non-*baumannii* *Acinetobacter* species acting as reservoirs of resistance genes.

4. To assess the suitability of high-throughput spectroscopic (FTIR) and spectrometric (MALDI-TOF MS) technologies for accurate, quick and low-cost *Acinetobacter* species identification and typing.

2.2. Outline of the thesis

This Thesis is organized in four chapters as follows:

Chapter 1 presents an overview of the state of the art regarding the topics of this Thesis, and is subdivided in five main sections. **Section 1.1.1** presents a brief summary of the history of *Acinetobacter* genus and respective taxonomic changes and an updated overview of the *Acinetobacter* species described until now. **Section 1.1.2** presents the main features and recent methodologies used for *Acinetobacter* species delineation and characterization. **Section 1.1.3** focuses on the distribution of *Acinetobacter* species in different ecological niches, such as human and non-human (e.g. environment, food and animals) sources. **Section 1.1.4** presents a little overview of the history behind the emergence and evolution of *Acinetobacter* spp., and also their clinical relevance. Finally, **Section 1.1.5** includes an overview concerning the virulence traits and the antibiotic resistance in *Acinetobacter* spp., mainly in *A. baumannii,* the molecular trends involving resistance to β-lactams (mainly carbapenems) and non-β-lactams (aminoglycosides, tetracyclines, fluoroquinolones and colistin) and also the population structure and mobile genetic elements involved in the current global scenario of MDR/XDR *A. baumannii*.

Chapter 2 includes the objectives and outline of the Thesis.

Chapter 3 presents the findings regarding the specific aims of the Thesis. Results from the experimental research performed yielded different research articles (n=9; 7 publications in peer review journals and 2 manuscripts in preparation). Such results have been organized throughout the papers, according to the following rationale:

Section 3.1. Carbapenem-resistant *Acinetobacter baumannii* clonal dynamics in Portuguese clinical settings explained by a highly successful interplay between antimicrobial resistance and virulence. Using the high-throughput technology WGS, the relevant traits (mainly related with antibiotic resistance and virulence potential) that may explain the dynamics of carbapenem-resistant *A. baumannii* clones in Portuguese clinical settings, in particular the replacement of ST208 by ST218, are analyzed. The data obtained is presented in the following research article:

Silva L, Ksiezarek M, Ramos H, Grosso F, Peixe L. Carbapenem-resistant

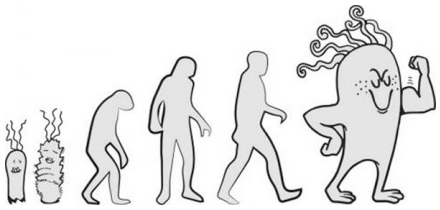
Acinetobacter baumannii clonal dynamics in Portuguese clinical settings explained by a successful interplay between antimicrobial resistance and virulence *(manuscript final draft).*

Section 3.2. Contribution of *non-baumannii Acinetobacter* species as reservoirs of resistance genes in clinical and non-clinical settings. The characterization of bla_{OXA-23} and bla_{OXA-58}-carrying *A. pittii* plasmids and bla_{IMP-5} *A. bereziniae* genetic environments from a Portuguese Hospital is conducted, exploring the role of non- *A. baumannii Acinetobacter* species in the dissemination of carbapenem resistance by horizontal gene transfer. Moreover, a study conducted in non-clinical niches of Benguela, Province of Angola, revealed a high diversity of *Acinetobacter* species, being some CHDL-producers, and a worrisome human exposition to these isolates. The data obtained is presented and organized in the following research articles:

- Grosso F, **Silva L**, Sousa C, Ramos H, Quinteira S, Peixe L. 2015. Extending the reservoir of bla_{IMP-5}: the emerging pathogen *Acinetobacter bereziniae*. Future Microbiology. DOI: 10.2217/fmb.15.88.
- **Silva L**, Grosso F, Branquinho R, Gonçalves-Ribeiro T, Sousa C, Peixe L. 2016. Exploring non-hospital-related settings of Angola reveals new *Acinetobacter* reservoirs for bla_{OXA-23} and bla_{OXA-58}. International Journal of Antimicrobial Agents. DOI: 10.1016/j.ijantimicag.2016.06.003.
- **Silva L**, Mourão J, Grosso F, Peixe L. 2017. Uncommon carbapenemase-encoding plasmids in the clinically emergent *Acinetobacter pittii*. Journal of Antimicrobial Chemotherapy. DOI: 10.1093/jac/dkx364.

Section 3.3. Developments on *Acinetobacter* species identification and typing. The potential of two high-throughput technologies, FTIR and MALDI-TOF MS, coupled with different bioinformatics tools, is evaluated for *Acinetobacter* species identification (species level) and *A. baumannii* characterization (infra-species level), in a large national and international collection of isolates. Moreover, we demonstrated the potential of FTIR-spectroscopy for depicting capsular (KL)-types. Of remark, the identification of capsular types more adapted for the survival in host (enriched in acidic sugars) for ST208 and ST218, the endemic clones more recently observed in our country and worldwide, that may also contribute for their great success. The results obtained are presented and organized in the following research articles:

- Sousa C, **Silva L**, Grosso F, Nemec A, Lopes J, Peixe L. 2014. Discrimination of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex species by Fourier transform infrared spectroscopy. European Journal of Clinical Microbiology and Infectious Diseases. DOI: 10.1007/s10096-014-2078-y.
- Sousa C, Botelho J, **Silva L**, Grosso F, Nemec A, Lopes J, Peixe L. 2014. MALDI-TOF MS and chemometric based identification of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex species. International Journal of Medical Microbiology. DOI: 10.1016/j.ijmm.2014.04.014.
- Sousa C, **Silva L**, Grosso F, Lopes J, Peixe L. 2014. Development of a FTIR-ATR based model for typing clinically relevant *Acinetobacter baumannii* clones belonging to ST98, ST103, ST208 and ST218. Journal of photochemistry and photobiology B. DOI: 10.1016/j.jphotobiol.2014.02.015.
- Sousa C, Botelho J, Grosso F, **Silva L**, Lopes J, Peixe L. 2015. Unsuitability of MALDI-TOF MS to discriminate *Acinetobacter baumannii* clones under routine experimental conditions. Frontiers in Microbiology. DOI: 10.3389/fmicb.2015.00481.
- **Silva L**, Rodrigues C, Grosso F, Peixe L. The secret is on sugar: capsular type explains the discrimination of *Acinetobacter baumannii* clones by Fourier-transform Infrared (FT-IR) Spectroscopy and Multilocus Sequence Typing *(manuscript final draft).*



Chapter 3

Results and discussion

"A person who never made a mistake never tried anything new."

Albert Einstein

3.1. Carbapenem-resistant *Acinetobacter baumannii* clonal dynamics in Portuguese clinical settings explained by a highly successful interplay between antimicrobial resistance and virulence

3.1.1. Carbapenem-resistant *Acinetobacter baumannii* clonal dynamics in Portuguese clinical settings explained by a successful interplay between antimicrobial resistance and virulence.

Carbapenem-resistant *Acinetobacter baumannii* clonal dynamics in Portuguese clinical settings explained by a successful interplay between antimicrobial resistance and virulence

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Manuscript Final Draft

1. Introduction

Acinetobacter baumannii is a common cause of serious hospital and, less often, community-acquired difficult-to-treat infections with resistance to multiple antibiotics frequently occurring worldwide [1]. Initial studies on population structure and epidemiology of multidrug-resistant *A. baumannii* strains attributed their global spread to three major clonal lineages, referred as international clones (ICs)-I, -II, and -III, whose diversity was deepened with the use of multilocus sequence typing (MLST). In Portugal, for almost two decades, carbapenem-resistant *A. baumannii* has been associated with very few clones, with usually only one dominating in a particular period [2]. Previous studies allowed us to describe the substitution of OXA-58 producing-ST103 by the OXA-40 producing-ST98, and, after, the substitution of ST98 by the worldwide disseminated ST208, at least, until 2010 [2]. Considering last data [3], the prevalence of carbapenem-resistant *Acinetobacter* spp. causing healthcare associated infections is still very high in Portugal (≥ 50%), however, there is no updated information on the recent clones responsible for this panorama, as well as their main characteristics.

In the present study, we aimed to characterize the population structure of *A. baumannii* isolates recovered from Portuguese nosocomial and community settings (2010-2015), in order to identify key features (antibiotic susceptibility profile, antibiotic resistance genes and virulence traits) contributing for the emergence and proliferation of particular *A. baumannii* clones.

2. Material and methods

Bacterial isolates collection

This study included a collection of 177 *A. baumannii* isolates (2010-2015) from two hospitals (North, H; and Centre of Portugal, CB), a Long-Term Care facility (LTCF) and a community clinical laboratory (CT). Isolates were mainly recovered from respiratory secretions (n=44), urine (n=43), blood cultures (n=27) and tissues (n=15) from patients of different wards, mainly Intensive Care Unit (n=22), Surgery (n=22) and Medicine (n=19) (Table 1). Isolates were initially identified by the VITEK 2 system (Biomérieux) and further confirmed by $bla_{\text{OXA-51-like}}$ detection and *rpoB/gyrB* genes partial sequencing [4,5].

Susceptibility testing and carbapenemase detection

Antimicrobial susceptibility profile to different β-lactams, aminoglycosides, tetracyclines, quinolones and trimethoprim-sulfamethoxazole was assessed by standard disc diffusion, Etest (carbapenems) and broth microdilution (colistin) methods, according to EUCAST (www.eucast.org) and CLSI guidelines (www.clsi.org). Isolates were categorized as multidrug-resistant (MDR) or extensively drug-resistant (XDR) bacteria according to Magiorakos and colleagues [7]. Carbapenemase production was assessed by the Blue-Carba biochemical assay [8] and carbapenem-hydrolyzing class D β-lactamases (CHDLs) genes were searched by PCR/sequencing [9].

Whole genome sequencing (WGS)

From the above collection, 12 *A. baumannii* isolates (ST208, n=4; ST218, n=4; and sporadic STs, n=4), together with 4 additional isolates from a previously characterized collection and belonging to the old endemic Portuguese clones [ST98, n=2 (2002-2006); ST103, n=2 (2001-2004)] [2], were subjected to WGS. This selection process aimed to congregate isolates from different STs, collected from different settings, over a long period of time (2001-2015). Total DNA was extracted using the Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI), according with the manufacturer´s instructions. DNA concentration was estimated using the Qubit dsDNA HS Assay Kit and the Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, USA). Extracted DNA was then sequenced with a standard 2x125 paired-end runs protocol on Illumina HiSeq 2000 (Illumina, San Diego, CA, USA). The quality of the high-throughput sequence data was assessed by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw sequence reads were then *de novo* assembled using SPAdes 3.9.0 (http://bioinf.spbau.ru/spades), and the quality was assessed by QUAST (http://quast.bioinf.spbau.ru). Additionally, 16 *A. baumannii* genome sequences of clinical strains isolated in different countries and belonging to the same STs found in our collection (except for ST98 and ST103 for which no genomes were available), were downloaded from NCBI database (https://www.ncbi.nlm.nih.gov/genome/403) for comparison purposes (accession numbers in Table S1).

Screening of antibiotic resistance and virulence genes

The distribution and richness of several genes previously associated with antimicrobial resistance and virulence in *A. baumannii* was determined across the studied genomes using the ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) and the Virulence Factors Database (VFDB) of pathogenic bacteria

(http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Acinetobacter). This analysis included acquired genes associated with resistance to aminoglycosides, β-lactams, macrolides, fluoroquinolones, sulphonamides, trimethoprim, tetracyclines and phenicol. The virulence factors screened included genes related to adherence (outer membrane protein A - *ompA*); biofilm formation [bacterial adhesin *bap*, usher-chaperone assembly system - *csuA/BABCDE* for pili production (which is regulated by the two-component system sensor kinase and response regulator (*bfmRS* genes), and *pgaABCD* locus for the biofilm exopolysaccharide matrix production]; iron uptake (*basABCDEFGHIJ*, *barAB* and *bauABCDEF* gene clusters for acinetobactin biosynthesis and release); lipooligosaccharide production (*lpsB* and *lpxC*); enterolisin E production (*entE*); heme oxygenase production (*hemO*); bacterial phospholipases production (*plcC* and *plcD* genes) and penicillin-binding protein 7/8 (PBP-7/8) production (*pbpG*).

Strain typing

Clonality of the 177 isolates was initially investigated by Fourier-Transform Infrared (FTIR) spectroscopy with attenuated total reflectance (ATR) as previously described [10,11]. Strain diversity was also assessed by MLST (Oxford scheme), using the sequence and profile definitions available at https://pubmlst.org/abaumannii/ and WGS [platform MLST 1.8 of Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/MLST/].

3. Results and discussion

The main characteristics of the recent collection of 177 *A. baumannii* isolates included in this study are summarized in Table 1. The ST218 was the most disseminated clone among healthcare facilities (115/177; 65%), being detected in several wards of both hospitals (H and CB) and in the community (CT) during the analysed period. The ST208 (single locus variant of ST218), endemic in our country between 2006-2010 [12], was only observed in hospital H and associated with a decreasing trend over the years (44/177; 25%; data not shown). All isolates belonging to ST218 and all but one isolate belonging to ST208 were Blue-Carba positive (OXA-23 producers), classified as XDR, and presented non-susceptibility to the β-lactams tested, including carbapenems (MIC>32 mg/L, Table 1). These antibiotic resistance profiles explain, to a large extend, the success of ST208 strains throughout the world (e.g. USA, Egypt, China, Thailand, Japan, Russia, Czech Republic, Australia, Denmark, Iraq and Switzerland (Oxford database). However, ST218, until now less

widespread, but already found in Asia (Saudi Arabia, Japan, Singapore, China [13] and Europe (Denmark, Spain and United Kingdom) [14,15], presented higher levels of aminoglycoside resistance, which seems to be due to a combination of factors: higher production of aminoglycoside modifying enzymes, higher frequency of the 16S ribosomal RNA methyltransferase ArmA enzyme (Table S1) and also by the great facility of this lineage to develop aminoglycosides heteroresistance, a phenomenon very difficult to detect in clinical laboratories, that contributes for therapeutic failure when the antibiotic is administered [16]. All ST218 genomes studied revealed the presence of the β-lactamases *bla*$_{\text{OXA-51-like}}$ (*bla*$_{\text{OXA-66}}$), *bla*$_{\text{OXA-23}}$ [flanked by two copies of the IS*Aba1* insertion element (Tn*2006*; chromosomal location)] and *bla*$_{\text{ADC-like}}$ (*bla*$_{\text{ADC-30}}$, n=4; *bla*$_{\text{ADC-25}}$, n=4), and a higher number of acquired antimicrobial resistance genes (e.g. *aacA4*, *aadA1*, *aph(3´)*, *armA*, *msr(E)*, *mph(E)*, *sul1*, *aac(6´)Ib-cr* or *catB8*), when compared with ST208 isolates (Table S1). This finding may contribute to the exchange of ST208 by ST218 observed in the last years in Portugal. Both clones revealed genomes with a medium size of 4.0Kb and 38.9% of GC content and no plasmids carrying antibiotic-resistance genes were predicted. Similarly, differences in antimicrobial resistance genes content between the oldest clones identified in Portugal clinical settings were observed, with ST98 displaying advantages over ST103 (e.g. *bla*$_{\text{OXA-40}}$, which confers higher levels of carbapenem-resistance than *bla*$_{\text{OXA-58}}$, *strA* and *strB* genes, that confers resistance to aminoglycosides, and also the *tet(B)* gene, for tetracycline resistance) that could justify the dominance of ST98 over ST1103. Sporadic STs (18/177; 10%; e.g. ST234, ST552, ST1557, ST1558) were more susceptible to antibiotics (confirmed by the lack of several resistance genes when genomes were analysed, Table S1), with only one isolate, from LTCF, being Blue-Carba positive due to the production of OXA-24/40. This carbapenemase gene, similarly to previously described among older Portuguese isolates, was plasmid-located (pMMCU3; pAc92, 99%Ident) and flanked by the typical XerC/XerD-like binding sites. However, the ST of this isolate (ST1557) was not even related to ST98 [17].

In what concerns to virulence genes, some of them were detected in all *A. baumannii* isolates (regardless of the lineage), suggesting their significant roles in the pathogenesis of *A. baumannii*. These genes included the adherence-related *ompA*, the surface-adhesion *bap*, and the serum resistance-related *pbpG* gene [18]. Although XDR *A. baumannii* clonal lineages showed common virulence traits (Table S2), the ST218 lineage exhibited an enriched virulence profile when compared to ST208, mainly because of the presence of an important gene (*hemO*) that codifies for heme oxygenase, essential for uptake and utilization of iron from their mammalian

hosts [19]. Moreover, all of the dominant clones (ST103, ST98, ST208 and ST218) showed significantly more virulence traits than the sporadic ones [e.g. the *pgaABCD* locus for the biofilm exopolysaccharide matrix production and the gene clusters responsible for iron uptake (*basABCDEFGHIJ*, *barAB* and *bauABCDEF*)] - both present only in some of the endemic isolates) (Table S2). These results suggest that a positive correlation might exist between resistance and increased virulence, raising the question of the existence of high-risk clones among *A. baumannii*. This information could be useful for clinicians, in order to adjust treatment regimens based on the expected degree of virulence and the severity of the illness of the patient, and for the infection control specialists, through the adaptation of eradication protocols to specific clones.

4. Conclusions

In summary, this work provided relevant features for understanding the predominance of the ST208 and ST218 worldwide. Moreover, it also provides the potential causes for the *A. baumannii* clonal dynamics observed in Portuguese clinical settings, i.e., higher antibiotic resistance and an enlarged virulence potential. Moreover, the data presented here, by increasing the knowledge on features responsible for the circulation and persistence of these specific lineages throughout the world, may aid in future prevention and/or treatment strategies.

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5. References

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Table 1. Main characteristics of the 177 *A. baumannii* isolates included in this study (2010-2015).

Species (no.)	ST (Oxford scheme) (no.)	Origin		CHDLs	MIC range (mg/L)			Non-β-Lactam Resistance profile (%)
		Hospital/Ward(s) (no.)[a]	Samples (no.)		IMP	MER	Colistin	
***A. baumannii* (n=177)**	**218 (n=115)**	**H**/Med (13), ICU (12), SUR (15), ED (7), MIU (11), Transplant unit (6), Nephrology (4), Urology (3), OC (4), CH (3), Physiatry (3), TBI (3), Endocrinology (3), Gastroenterology (2), others (7), NK (2)	**H**/Urine (24), respiratory tract (22), blood (15), tissues (7), exudate (3), pus (6), drainage fluid (4), cerebrospinal fluid (4), catheter (3), peritoneal liquid (2), bile (2), others (6)	*bla*$_{OXA-66}$; *bla*$_{OXA-23}$ (n=115)	> 32	> 32	0.25-8	AK (80.9), GM (86.1), TB (87), KM (87), NT (90.4), TE (100), MH (64.3), TG (64.3), CP (100), SX (97.4), TZP (100)
		CT/Community (6)	**CT**/Exudate (4), urine (2)				0.5-8	
		CB/NK (11)	**CB**/Respiratory tract (6), urine (3), blood (1), tissues (1)				0.5-1	
	208 (n=44)	**H**/ICU (8), Med (6), SUR (5), OC (4), ED (4), Others (10), NK (6)	Respiratory tract (13), urine (8), blood (8), tissues (5), exudate (4), drainage fluid (2), pus (1), cerebrospinal fluid (1), NK (1)	*bla*$_{OXA-66}$; *bla*$_{OXA-23}$ (n=43)	> 32	> 32	0.25-16	AK (14), GM (58.1), TB (21), KM (4.7), NT (79.1), TE (100), MH (95.3), TG (83.7), CP (100), SX (100), TZP (100)
		NK (1)	Urine (1)	*bla*$_{OXA-51-like}$ (n=1)	1.5	0.25	0.5	TE, TZP
	Sporadic STs (n=18)	**H**/ED (3), ICU (2), OC (2), SUR (2), PED (1), CAR (1), CH (1), ORL (1), NK (4)	Urine (6), Respiratory tract (3), blood (3), tissues (2), exudate (2), pus (1)	*bla*$_{OXA-51-like}$ (n=14); *bla*$_{OXA-91}$ (n=1); *bla*$_{OXA-120}$ (n=1); *bla*$_{OXA-338}$ (n=1)	0.38 - 0.75	0.38 - 1.0	0.125-1	AK (5.9), GM (5.9), TB (5.9), KM (5.9), NT (5.9), TG (11.8), CP (11.8), SX (5.9)
		LTCF/- (1)	Colonization sample (1)	*bla*$_{OXA-104}$; *bla*$_{OXA-24/40}$	> 32	> 32	4	CP, TZP

[a] **H**, Hospital located at North of Portugal; **CT**, Community clinical laboratory; **CB**, Hospital located at Centre of Portugal; **LTCF**, Long-term care facility at North of Portugal; **CAR**, Cardiology; **CH**, Clinical hematology; **ED**, Emergency department; **ICU**, Intensive care unit; **Med**, Medical unit(s) A, B, C and D; **MIU**, Medical intermediate unit; **OC**, Outpatient consultation; **PED**, Pediatrics; **ORL**, Otorhinolaryngology; **SUR**, Surgery; **TBI**, Traumatic brain injury unit; **NK**, Origin not known

Abbreviations: AK, amikacin; **CAZ**, ceftazidime; **CP**, ciprofloxacin; **CRO**, ceftriaxone; **FEP**, cefepime; **GM**, gentamicin; **IPM**, imipenem; **KM**, kanamycin; **MEM**, meropenem; **MH**, minocycline; **NT**, netilmicin; **SX**, trimethoprim/sulfamethoxazole; **TB**, tobramycin; **TE**, tetracycline; **TG**, tigecycline; **TZP**, piperacillin/tazobactam.

Table S1. Antibiotic resistance genes distribution in the *A. baumannii* strains of different lineages. Isolates marked with an asterisk were downloaded from Genbank database.

		β-lactams				Aminoglycosides							MLS		SDI			FQ	Phenicol	TCN	Accession number
		bla$_{ADC-like}$	Acquired *bla*$_{OXA-like}$	*bla*$_{OXA-51-like}$	*bla*$_{TEM-like}$	*aacA4*	*aac(3)*	*aadA1;aadB*	*aph(3´)*	*armA*	*strA*[*1]	*strB*[*2]	*msr(E)*	*mph(E)*	*sul1*	*sul2*	*dfrA1*	*aac(6´)Ib-cr*	*catB8*	*tet(B)*	
ST103 (2001-2004)	**Ac23**	*bla*$_{ADC-1}$	*bla*$_{OXA-58}$	*bla*$_{OXA-132}$				*aadA1;aadB*	*aph(3')-VIa*												[2]
	Ac246	*bla*$_{ADC-2}$	*bla*$_{OXA-58}$	*bla*$_{OXA-132}$	*bla*$_{TEM-116}$			*aadA1;aadB*	*aph(3')-IIa*												[2]
ST98 (2002-2006)	**Ac55**	*bla*$_{ADC-1}$	*bla*$_{OXA-40}$	*bla*$_{OXA-66}$	*bla*$_{TEM-116}$		*aac(3)-IIa*		*aph(3´)-IIa; aph(3´)-VIa*												[2]
	H25	*bla*$_{ADC-1}$	*bla*$_{OXA-40}$	*bla*$_{OXA-66}$					*aph(3´)-VIa*												[2]
ST208 (2010-2015	**H466**	*bla*$_{ADC-30}$	*bla*$_{OXA-23}$	*bla*$_{OXA-66}$	*bla*$_{TEM-1D}$																This study
	H580	*bla*$_{ADC-30}$	*bla*$_{OXA-23}$	*bla*$_{OXA-66}$	*bla*$_{TEM-1D}$																This study
	H603	*bla*$_{ADC-30}$	*bla*$_{OXA-23}$	*bla*$_{OXA-66}$	*bla*$_{TEM-1D}$																This study
	H678	*bla*$_{ADC-30}$	*bla*$_{OXA-23}$	*bla*$_{OXA-66}$	*bla*$_{TEM-1D}$				*aph(3´)-IIa*												This study
	1552389*	*bla*$_{ADC}$		*bla*$_{OXA}$		*aac(6´)*															JFCS00000000.1

	β-lactams				Aminoglycosides						MLS		SDI			FQ	Phenicol	TCN	Accession number
	bla$_{\text{AD}}$ DC-like	Acquired *bla*$_{\text{OXA}}$-like	*bla*$_{\text{OX}}$ XA-51-like	*bla*$_{\text{TE}}$ M-like	*aacA4*	*aac(3)*	*aadA1/aadB*	*aph(3´)*	*arm A*	*str A*[*1*]	*strB*[*2*]	*msr(E)*	*mph(E)*	*sul1*	*sul2*	*dfrA1*	*aac(6´)Ib-cr*	*catB8*	*tet(B)*	
1146103*	C-82		A-82		*-I*															
	bla$_{\text{AD}}$ C-30		*bla*$_{\text{OX}}$ A-66	*bla*$_{\text{TE}}$ M-1D	*aac(6´)-I*	*aac(3)-Ia*		*aph(3´)-Ia*												JMNS00000000.1
CI77*	*bla*$_{\text{AD}}$ C-30		*bla*$_{\text{OX}}$ A-66	*bla*$_{\text{TE}}$ M-1D				*aph(3´)-Ia*												AVOC00000000.1
Perm*	*bla*$_{\text{AD}}$ C-30		*bla*$_{\text{OX}}$ A-66																	AUZL00000000.1
MRY09-0642*	*bla*$_{\text{AD}}$ C-30		*bla*$_{\text{OX}}$ A-82		*aac(6´)-Ib*															BASA00000000.1
MRY12-0277*	*bla*$_{\text{AD}}$ C-30		*bla*$_{\text{OX}}$ A-66		*aac(6´)-Ib*	*aac(3)-Ia*														BASC00000000.1
48055*	*bla*$_{\text{AD}}$ C-30-like	*bla*$_{\text{OXA-23}}$	*bla*$_{\text{OX}}$ A-66	*bla*$_{\text{TE}}$ M-1D	*aac(6´)-II*	*aac(3)-I*		*aph(3´)-Ia; aph(3´)-VIa*												AOSP00000000.1
AB1H8*	*bla*$_{\text{AD}}$ C-25		*bla*$_{\text{OX}}$ A-66	*bla*$_{\text{TE}}$ M-1D		*aac(3)-Ia*		*aph(3´)-Ia*											*tet(B)+tet(39)*	ANNC00000000.1
T7*	*bla*$_{\text{AD}}$ C-73	*bla*$_{\text{OXA-23}}$	*bla*$_{\text{OX}}$ A-66	*bla*$_{\text{TE}}$ M-1D				*aph(3´)-Ia*												JRQT00000000.1
NIPH2061*	*bla*$_{\text{AD}}$ C-30		*bla*$_{\text{OX}}$ A-109-like					*aph(3´)-VIa*												APOW00000000.1
BIDMC56*	*bla*$_{\text{AD}}$ C-30		*bla*$_{\text{OX}}$ A-113	*bla*$_{\text{TE}}$ M-1D	*aac(6´)-Ib*															JMUH00000000.1
AB-908-12*	*bla*$_{\text{AD}}$ C-30		*bla*$_{\text{OX}}$ A-113	*bla*$_{\text{TE}}$ M-1D																AMHV00000000.1

		β-lactams				Aminoglycosides							MLS		SDI			FQ	Phenicol	TCN	Accession number
		bla$_{AD}$	Acquired *bla*$_{OXA-like}$	*bla*$_{OX}$	*bla*$_{TE}$	*aacA4*	*aac(3)*	*aadA1/aadB*	*aph(3´)*	*armA*	*strA*[*1]	*strB*[*2]	*msr(E)*	*mph(E)*	*sul1*	*sul2*	*dfrA1*	*aac(6´)Ib-cr*	*catB8*	*tet(B)*	
		DC-like		XA-51-like	M-like																
ST218 (2010-2015	**H482**	*bla*$_{AD}$ C-30	*bla*$_{OXA-23}$	*bla*$_{OX}$ A-66																	This study
	H202	*bla*$_{AD}$ C-30	*bla*$_{OXA-23}$	*bla*$_{OX}$ A-66		*aac(6´)-Ib*			*aph(3´)-Ia*												This study
	H631	*bla*$_{AD}$ C-30	*bla*$_{OXA-23}$	*bla*$_{OX}$ A-66	*bla*$_{TE}$ M-116	*aac(6´)-Ib*			*aph(3´)-Ia*												This study
	H735	*bla*$_{AD}$ C-30	*bla*$_{OXA-23}$	*bla*$_{OX}$ A-66																	This study
	AB210*	*bla*$_{AD}$ C-25	*bla*$_{OXA-23}$	*bla*$_{OX}$ A-66		*aac(6´)-Ib*			*aph(3´)-Ia*												AEOX00000000.1
	W7282*	*bla*$_{AD}$ C-25	*bla*$_{OXA-23}$	*bla*$_{OX}$ A-66		*aac(6´)-Ib*			*aph(3´)-Ia*												AIEH00000000.1
	S46*	*bla*$_{AD}$ C-25	*bla*$_{OXA-23}$	*bla*$_{OX}$ A-66		*aac(6´)-Ib*			*aph(3´)-Ia*												CP002522.2
	S19*	*bla*$_{AD}$ C-25	*bla*$_{OXA-23}$	*bla*$_{OX}$ A-66		*aac(6´)-Ib*			*aph(3´)-Ia*												LAIN00000000.1
Sporadic STs (2010-2015	**316.1**		*bla*$_{OXA-24}$	*bla*$_{OX}$ A-104	*bla*$_{TE}$ M-116																This study
	H745	*bla*$_{AD}$ C-52		*bla*$_{OX}$ A-91																	This study
	H637	*bla*$_{AD}$ C-156		*bla*$_{OX}$ A-120	*bla*$_{TE}$ M-116																This study
	H366			*bla*$_{OX}$ A-338	*bla*$_{TE}$ M-116																This study

Abbreviations: MLS, macrolide-lincosamide-streptogramin B; **FQ**, fluoroquinolone; **SDI**, sulphonamide; **TCN**, tetracycline. Grey coloured boxes mean presence of gene.[*1] Also called *aph(6)-Ia*; [*2] Also called *aph(6)-Id*. Grey indicates the gene presence, when applicable, the allelic variant is indicated.

Table S2. Virulence genes distribution in the *A. baumannii* strains of different lineages.

Abbreviations: ***ompA***, outer membrane protein A gene; ***bap***, bacterial adhesin gene; ***csuA/BABCDE***, usher-chaperone assembly system; ***bfmRS***, two-component system sensor kinase and response regulator; ***pgaABCD***, exopolysaccharide matrix production; ***basABCDEFGHIJ***, acinetobactin biosynthesis gene cluster; ***barAB*** and ***bauABCDEF***, acinetobactin release gene clusters; ***lpsB*** and ***lpxC***, lipopolysaccharide production genes; ***entE***, enterolisin E production gene; ***hemO***, heme oxygenase production gene; ***plcC*** and ***plcD***, bacterial phospholipases production genes; ***pbpG***, penicillin-binding protein 7/8 production gene. Grey coloured boxes mean presence of complete gene clusters.

3.2. Contribution of non-*baumannii* *Acinetobacter* species as reservoirs of resistance genes in clinical and non-clinical setting

3.2.1. Extending the reservoir of $bla_{\text{IMP-5}}$: the emerging pathogen *Acinetobacter bereziniae*.

3.2.2. Exploring non-hospital-related settings of Angola reveals new Acinetobacter reservoirs for $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-58}}$.

3.2.3. Uncommon carbapenemase-encoding plasmids in the clinically emergent *Acinetobacter pittii*.

Extending the reservoir of *bla*$_{IMP-5}$: the emerging pathogen *Acinetobacter bereziniae*

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Extending the reservoir of bla_{IMP-5}: the emerging pathogen *Acinetobacter bereziniae*

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Aim: *Acinetobacter bereziniae* clinical relevance is starting to be recognized; however, very few descriptions of its carbapenem resistance currently exist. Here we characterize two carbapenem-resistant *A. bereziniae* isolates. **Materials & methods:** Isolates were obtained from environmental and clinical samples. Carbapenemases were searched by phenotypic, biochemical and PCR assays. Clonality was studied by ApaI-PFGE and genetic location for carbapenemase genes were assessed by I-CeuI and S1 hybridizations. **Results:** Isolates were not clonally related but both produced the 'exclusively Portuguese' IMP-5, with the clinical isolate also producing an OXA-58. The carbapenemase genes were plasmid located. **Conclusion:** Our results emphasize the role of non-*baumannii Acinetobacter* species as important reservoirs of clinically relevant resistance genes that could also contribute to their emergence as nosocomial pathogens.

KEYWORDS
- *Acinetobacter bereziniae*
- carbapenem resistance
- IMP-5 • integron
- metallo-β-lactamase
- oxacillinase

Metallo-β-lactamases (MBLs) currently represent an important and emerging mechanism of acquired-carbapenem resistance in Gram-negative bacteria, responsible for difficult-to-treat nosocomial infections, and are consequently associated with both high morbidity and mortality rates. Enzymes belonging to VIM and IMP families are among the most prevailing MBLs, rapidly growing and conferring the greatest clinical threat. Thus far, the IMP-5 MBL has only been described in Portugal, initially in an *Acinetobacter baumannii* clinical isolate belonging to the sequence type (ST) 120 and posteriorly in *Pseudomonas aeruginosa* clinical isolates [1–3]. The bla_{IMP-5} gene was described as part of the class 1 integron In*76*, located on a Tn*402*-like transposon, with two miniature inverted-repeat transposable elements (MITE)-like flanking the 5′CS to 3′CS regions [1,2]. This structure, also associated with other resistance determinants, such as bla_{PER-1} in *Acinetobacter johnsonii*, represents a mobilizable unit which might be responsible for intra- and interspecies transfer, via transposition or homologous recombination [2,3]. Notwithstanding, the reservoir of the 'exclusively Portuguese' bla_{IMP-5} gene is yet to be known.

Within the last decades, *Acinetobacter* species have, surprisingly and successively, evolved from traditionally harmless organisms into important nosocomial pathogens exhibiting high levels of antibiotic resistance, including extreme drug resistance [4,5]. *Acinetobacter bereziniae* (formerly

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Acinetobacter genomospecies 10) is presently considered one of the non-*baumannii Acinetobacter* emerging pathogenic species [6]. Descriptions of this species include vegetable samples [7], fecal carriage among healthy humans [8] and different human clinical specimens, suggesting its wide distribution [9]. More recently, it has also been associated with chronic obstructive pulmonary disease, urinary tract infection, pneumonia, sepsis and bacteremia [6,10–11]. Reports on carbapenem resistance in *A. bereziniae* were previously associated with the production of IMP-1 and IMP-19, SIM-1, VIM-2 and NDM-1 MBLs [10–13]. Additionally, two variants of the intrinsic oxacillinase OXA-228-like, OXA-229 and OXA-257, able to confer carbapenem resistance when overexpressed, were recently described in *A. bereziniae* [14,15].

This report aims the characterization of two *A. bereziniae* isolates obtained in the same hospital, from the water of a newborn incubator's humidity chamber and from a blood culture, which exhibited an unusual multidrug resistance phenotype, including resistance to carbapenems.

Materials & methods

Two *A. bereziniae* isolates, obtained at an interval of 4 years in the same University Hospital in Portugal, were studied. *Acinetobacter bereziniae* HGSA93 was collected in 2008 from the water reservoir of a new-born's incubator humidity system, while *A. bereziniae* HGSA593 was obtained in 2012 from a blood culture of a hospitalized patient with chronic renal disease. Isolates were initially identified as *Acinetobacter lwoffii* by the vitek 2 system. Further identification included *rpoB* gene partial sequencing [16] and PCR searching for *Acinetobacter* species-specific oxacillinase, OXA-228-like, using previously described primers [14].

Antibiotic susceptibility testing was performed by disc diffusion method and E-test following CLSI guidelines [17]. Carbapenemase production was assessed by the Blue-Carba test [18]. Furthermore, isolate crude extracts were used to conduct a bioassay with imipenem (with and without EDTA).

Acquired carbapenemase genes (MBL and carbapenem-hydrolyzing class D β-lactamases [CHDL]) were searched by PCR using primers and conditions as previously described [1,19], and confirmed by sequencing. Class 1 integrons were searched with primers directed for 5′CS and 3′CS regions [20] and confirmed by sequencing. Additionally, association of the MBL genes with the Tn*402*-like transposon (GenBank accession number JF810083) was investigated by PCR mapping with a combination of primers for the MBL gene [1], *orf5* [21] and MITEs sequences (MITE_Fw_2: 5′-GATAACCAATCCATTTATGACA-3′; MITE_Rv_2: 5′-TGACTGACCATTAAAGTCTCAA-3′).

The carbapenemase genes location was assessed by hybridization of I-*Ceu*I- and S1-digested genomic DNA with specific probes for the carbapenemases and 16S rRNA genes. Plasmid characterization was performed using the PCR-based replicon typing scheme for *A. baumannii* plasmids [22].

Transfer of carbapenemase genes was attempted by conjugation with rifampicin-resistant mutant of *A. baumannii* ATCC 17978 (kindly provided by Dr Ruth Hall), and by electrotransformation with the plasmid DNA from HGSA593 isolate, using *Acinetobacter baylyi* ADP1 (kindly provided by Dr German Bou), as the recipient strain. Selection was performed on Mueller-Hinton agar plates supplemented with imipenem (0.5 mg/l) and rifampicin (100 mg/l) for conjugation assays and only with imipenem (0.5 mg/l) for electrotransformation. Presumptive transformants were confirmed by searching carbapenemases genes by PCR.

Clonal relatedness for the two isolates was assessed by genomic DNA macrorestriction with

Table 1. Molecular characterization of *A. bereziniae* isolates included in this study.

Isolate	Year	Source	Carbapenemase genes (plasmid size, Kb)	Class 1 integron content (size, Kb)	Antibiotic resistance profile (MIC, mg/l)
HGSA93	2008	Water from a newborn incubator humidity system	bla_{IMP-5} (~310)	bla_{IMP-5} (1.5)	IMP (>32); MEM (>32); ATM (32); FEP (>32); CAZ (>256); PTZ (>256); K
HGSA593	2012	Blood culture (inpatient with chronic renal disease)	bla_{IMP-5} (~200), bla_{OXA-58} (~90)	bla_{IMP-5}- bla_{OXA-4} (2.5), *aacA4* (1.2)	IMP (**>32**); MEM (**>32**; 12); ATM (**32**); FEP (**32**); CAZ (**>256**); PTZ (**>256**); K; CN; TOB

β-lactam MICs are presented; resistance transferred by electrotransformation is bold (when only bla_{OXA-58} was transferred) or bold and underlined (when both bla_{IMP-5} and bla_{OXA-58} were transferred).
ATM: Aztreonam; CAZ: Ceftazidime; CN: Gentamicin; FEP: Cefepime; IMP: Imipenem; K: Kanamycin; MEM: Meropenem; PTZ: Piperacillin-tazobactam; TOB: Tobramycin.

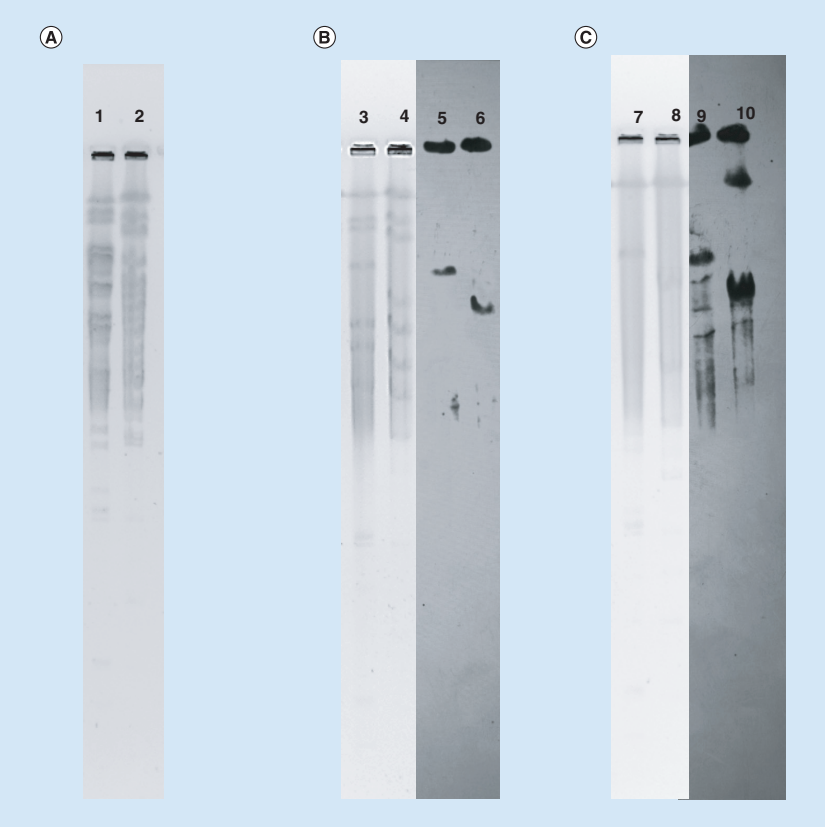

Figure 1. Apa I-, I-Ceu I- and S1-PFGE patterns from *A. bereziniae* isolates. (A) Apa I-PFGE, lane 1 HGSA93, lane 2 HGSA593; **(B)** I-Ceu I- PFGE and respective southern blot, lane 3 HGSA93, lane 4 HGSA593, lane 5 HGSA93 IMP-5 hybridization, lane 6 HGSA593 IMP-5 hybridization; S1-PFGE and respective Southern blot, lane 7 HGSA93, lane 8 HGSA593, lane 9 HGSA93 IMP-5 hybridization, lane 10 HGSA593 IMP-5 hybridization.

*Apa*I followed by pulsed-field gel electrophoresis (PFGE).

Results & discussion

HGSA93 and HGSA593 isolates belonged to *A. bereziniae* species, according to the *rpoB* partial gene sequencing (with both displaying 98% nucleotide identity with *A. bereziniae* type strain CIP 70.12^T, GenBank accession number DQ207475.1). The $bla_{OXA-228}$, an intrinsic oxacillinase gene from *A. bereziniae* species, was detected by PCR (100% homology with $bla_{OXA-228}$ gene described in *A. bereziniae* strain CIP 70.12^{T}GenBank accession number JQ422053.1) and presented the same promoter sequences (TTCAAT and TGGTAT for the -35 and -10 sequences, respectively) that have been related with its low expression [14,15].

Both isolates presented an unusual antibiotic resistance profile that included resistance to all tested β-lactams. HGSA93 remained susceptible to aminoglycosides (except kanamycin), while HGSA593 was only susceptible to amikacin **(Table 1)**. Blue-carba test revealed the presence of carbapenemase activity and the bioassay with EDTA strongly suggested the presence of an MBL, further identified as IMP-5 in both isolates by PCR and sequencing. Search for acquired CHDL revealed that HGSA593 also harbored bla_{OXA-58}, which is a carbapenemase that had

been endemic within this hospital among *A. baumannii* clinical isolates and disappeared after 2006 [5]. Sequencing of the bla_{IMP-5}-encoding class 1 integron, from HGSA93, revealed 100% homology with the previously described In*76* [1] and its nucleotide sequence was deposited in the GenBank database (accession number KF732850.1). PCR mapping showed that this genetic platform was also flanked by MITE structures, a fact that might justify bla_{IMP-5} gene mobilization [2,3]. Curiously, sequence analysis indicated the presence of a weak -35 promoter (TTGACA instead of TTGATA) upstream bla_{IMP-5} gene [23], contrary to what was expected by the observed high carbapenem resistance level **(Table 1)**. HGSA593 harbored similar genetic vicinity for bla_{IMP-5}, but the integron content included an extra gene cassette (bla_{OXA-4}) downstream bla_{IMP-5} gene. Moreover, HGSA593 harbored another class 1 integron carrying an *aacA4* gene, which might explain its additional resistance to aminoglycosides. I-*Ceu*I- and S1 hybridizations revealed that bla_{IMP-5} gene was located in a ~310 Kb plasmid for HGSA93 and in a ~200 Kb plasmid for HGSA593 **(Figure 1)** and bla_{OXA-58} was located in a ~90 Kb additional plasmid. These plasmids were untypeable using the current replicon typing scheme [22], which is now commonly reported among non-*baumannii Acinetobacter* species [24]. Conjugation attempts were unsuccessful, but plasmids from HGSA593 were transferred and expressed in *A. baylyi* ADP1, with transformants exhibiting resistance to β-lactams, including carbapenems **(Table 1)**.

Curiously, *Apa*I-PFGE assay revealed that *A. bereziniae* isolates were unrelated **(Figure 1)**, suggesting that this species might easily acquire different plasmids and act as reservoir of clinically relevant resistance genes.

Conclusion

Our data highlight the ability of non-*baumannii Acinetobacter* species to acquire relevant resistance genes which could contribute for their emergence as nosocomial pathogens. Furthermore, cumulative findings suggest that different species of *Acinetobacter* seem to constitute important reservoirs of resistance genes outside clinical settings. In this study, although the first *A. bereziniae* isolate was not associated with any infection episode, its detection in the newborn's incubator humidity chamber, along with the second bloodstream isolate and previous works on this species, should indicate the possibility of 'environmental' species emerge as etiologic agents of nosocomial infections, particularly within vulnerable populations.

Finally, we believe that further studies focused on the local microbial ecology of carbapenemase producers are imperative for the full understanding of the observed endemicity of IMP-5 in our country and for the effective prevention of its dissemination.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

EXECUTIVE SUMMARY

- We extended the species reservoir of the 'exclusively Portuguese' IMP-5 carbapenemase.
- Environmental *Acinetobacter bereziniae* isolates could act as reservoirs for bla_{IMP-5}.
- Newborn incubator systems could be a source of carbapenemase producing *Acinetobacter* spp.
- We confirm the pathogenic potential of carbapenem-resistant *A. bereziniae*.

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Exploring non-hospital-related settings of Angola reveals new Acinetobacter reservoirs for *bla*$_{OXA-23}$ and *bla*$_{OXA-58}$

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mcr-1-mediated colistin resistance would force strains to rapidly eradicate from the normal gut flora. Previous reports on short-term colonisation of colistin-resistant *E. coli* are consistent with this [3]. However, travel and the constant influx of new and better adapted strains is a continuous cause of concern.

Presently, ESBL resistance or other co-resistance was not linked to pIP2-01, but as an IncI2 plasmid in *E. coli* our biggest fear is that ESBLs and *mcr-1* evolutionary paths converge and together find their way to nosocomial species, possibly causing severe outbreaks in the future. Analyses made in retrospect indicate that *mcr-1* alone or in co-location with ESBLs are present, although undetected, in most continents [4]. So far, the earliest report is of an *mcr-1*-positive Chinese chicken isolate dating from the 1980s [5].

In conclusion, we report the first detection of plasmid-mediated *mcr-1* in Norway. This finding adds to the documentation of the global dissemination of plasmid-mediated *mcr-1*, including spread via normal flora *E. coli*. The origin of pIP2-01 remains uncertain, although the patient's travel history substantiates import from India. This observation establishes the presence of *mcr-1*-harbouring plasmids in Norway since 2014.

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Exploring non-hospital-related settings in Angola reveals new *Acinetobacter* reservoirs for bla_{OXA-23} and bla_{OXA-58}

Sir,

Infections caused by carbapenem-resistant *Acinetobacter* spp. have a significant impact on patient morbidity and mortality. Carbapenem resistance arises mostly by the production of carbapenem-hydrolysing class D β-lactamases (CHDLs), which are not limited to *Acinetobacter baumannii* but also occur in other *Acinetobacter* spp. increasingly associated with human infections [1]. However, little is known about the presence of CHDLs and their origins and reservoirs among *Acinetobacter* from non-clinical sources. Furthermore, in Africa, available data regarding *Acinetobacter* epidemiology and carbapenem resistance are limited to *A. baumannii* clinical isolates mainly from northern countries [2]. Angola, a sub-Saharan country with close relations with Europe, Asia and South America, is characterised by poor sanitary conditions that increase the population's vulnerability, currently reflected by the record numbers of malaria and yellow fever (http://www.cdc.gov/yellowfever/maps/africa.html).

In this study, the occurrence, diversity and antimicrobial resistance, in particular to carbapenems, of *Acinetobacter* spp. from non-clinical sources from Benguela Province (Angola) was evaluated as part of a larger surveillance project on bacterial resistance to clinically relevant antibiotics [3].

From 63 samples [3] collected in different communes of Benguela Province, 29 revealed the presence of *Acinetobacter*, including rectal swabs from healthy volunteers (8/18), aquatic environments (river, human drinking water, wastewater; 8/20), rectal swabs from healthy animals (calf/cows; 3/10) and their respective environments (water, feed, floor/walls; 7/8) from four different farms, and drinking water from a wild park (3/3) (Supplementary Table S1). The remaining samples (rectal swabs from monkeys and goats) were negative for *Acinetobacter*. Presumptive *Acinetobacter* isolates ($n = 73$) recovered from CHROMagar™ Orientation with/without cefotaxime (1 mg/L), ceftazidime (1 mg/L) or imipenem (1 mg/L) were further identified using a Bruker Biotyper® matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) system as well as *rpoB*/*gyrB* analysis [1,4]. Ten different *Acinetobacter* spp. were identified, with MALDI-TOF/MS results being congruent with *rpoB*/*gyrB*, except for *A gerneri*, *A. bereziniae* and *A. pittii*, first identified as *Alcaligenes faecalis*, *A. guillouiae* and *A. baumannii*, respectively. In addition, MALDI-TOF/MS identified three isolates as *A. towneri*, however *rpoB*/*gyrB* revealed only 92–93% nucleotide similarity with this species, being given the provisional name of *A. towneri*-like sp.

A. baumannii was the most predominant species (26/73) (Supplementary Table S1). Interestingly, some particular clones established by pulsed-field gel electrophoresis (PFGE) with the restriction enzyme *Apa*I (Supplementary Table S1) were detected both from healthy volunteers and water samples (untreated wastewater, river and drinking water for animals), raising the possibility of a water–human cycle of transmission. *A. pittii* was the second most prevalent species (17/73) and, to our knowledge, was identified for

Table 1
Characterisation of carbapenemase-producing *Acinetobacter* spp. isolates.

Species (*n*)	Origin/sample	Commune[a]	PFGE type[b]	Acquired CHDL and genetic environment	CHDL gene location	Carbapenem MIC (mg/L)				Resistance to other antibiotics	Concomitant resistant bacteria (*n*)[d]
						Parent		Transformant			
						MEM	IPM[c]	MEM	IPM		
A. johnsonii (1)	Drinking water for humans/56	Baía Farta	Ajo_I	bla_{OXA-58} (IS*Aba3* downstream)	Ca. 75 kb untypable plasmid[e]	1.5	1.5	**2.0**	**4.0**[f]	FEP, CAZ, CRO, **TET**, CIP	–
A. towneri-like sp. (3)[g]	Lobito river water/39	Lobito	At_I	bla_{OXA-23} (IS*Aba1* upstream)	Chromosome	1	3	NA	NA	–	*Klebsiella pneumoniae* with SHV-12 (1)
	Untreated wastewater from a treatment station/45	Benguela	At_{II}	bla_{OXA-23} (IS*Aba1* upstream)	Chromosome	1.5	1.5	NA	NA	FEP	–
	Wastewater from an urban sewer line/58	Catumbela	At_{III}	bla_{OXA-23} (IS*Aba1* upstream)	Chromosome	1.5	2	NA	NA	–	*Klebsiella oxytoca* with $bla_{CTX-M-15}$ (1)

PFGE, pulsed-field gel electrophoresis; CHDL, carbapenem-hydrolysing class D β-lactamase; MIC, minimum inhibitory concentration; MEM, meropenem; IPM, imipenem; FEP, cefepime; CAZ, ceftazidime; CRO, ceftriaxone; TET, tetracycline; CIP, ciprofloxacin; NA, not applicable.
[a] Samples were collected from Benguela Province, which comprises nine municipalities (Benguela, Lobito, Bocoio, Balombo, Ganda, Cubal, Caimbambo, Baía Farta and Chongorói), divided into more than 30 communes.
[b] Clonality was established by *Sma*I (for *Acinetobacter junii* and *A. johnsonii*) or *Apa*I (for other *Acinetobacter* spp.) PFGE. Pattern analysis was performed by InfoQuest with PFGE types comprising isolates with *Sma*I/*Apa*I patterns presenting similarity levels >74.5%.
[c] MICs higher than the epidemiological cut-off value (ECOFF) (1 mg/L) but lower than the clinical resistance breakpoint (2–8 mg/L) or within intermediate susceptibility clinical range in *A. towneri*-like (sample 39).
[d] Ribeiro et al [3].
[e] Untypeable by the current *Acinetobacter* replicon typing scheme [1].
[f] MIC higher than that observed in the donor strain.
[g] New primers were designed for *gyrB A. towneri*-like sp. isolates amplification and sequencing: gyrB_AcT1_F, GTGGCCTAGATGCTGTTCGT; gyrB_AcT1_R, TTACCCGCGATAGATTTTGC; gyrB_AcT2_F, AAGGTTTAACCGCGATTGTG; and gyrB_AcT2_R, GGCCAATACGTTGCAGATTT.
Antimicrobial resistance transfer by transformation appears in bold.

the first time in rivers, wastewater and healthy farm animals (Supplementary Table S1). Farms were the most enriched environment, presenting a high variety of *Acinetobacter* spp.

Susceptibility to different β-lactams, aminoglycosides, tetracyclines and quinolones was determined by disk diffusion and Etest methods according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) guidelines, with the majority of isolates showing reduced susceptibility to at least one of the antimicrobial agents, mostly β-lactams (Supplementary Table S1). Carbapenemases were searched for by the Blue-Carba assay and PCR for all isolates [1]. Four isolates were Blue-Carba-positive, presenting an imipenem minimum inhibitory concentration (MIC) greater than the epidemiological cut-off value (ECOFF) but lower than the clinical resistance breakpoint (Table 1). PCR assays identified bla_{OXA-23} among *A. towneri*-like isolates ($n = 3$) and bla_{OXA-58} in one *A. johnsonii* isolate (Table 1). Curiously the OXA-23-producing *A. towneri*-like isolates were clonally unrelated, obtained from Lobito River and untreated wastewater (20 km distant). Moreover, these samples harboured additional resistant bacteria (Table 1) [3]. The OXA-58-producing *A. johnsonii* was obtained from treated tap water also positive for *A. baumannii* and *A. pittii*, suggesting an important role for water in the transmission of *Acinetobacter* spp. to humans in this region. To our knowledge, this is the first evidence of OXA-58-producing multidrug-resistant *A. johnsonii* isolates from a non-related-hospital source. I-*Ceu*I- and S1-PFGE hybridisations [1] revealed the chromosomal location of bla_{OXA-23} and the association of bla_{OXA-58} with an ca. 75-kb untypable plasmid. The bla_{OXA-58} transformants, obtained by electrotransformation of a rifampicin-resistant mutant *A. baumannii* ATCC 17978 strain, acquired resistance to β-lactams, particularly imipenem (MIC = 4 mg/L), and to tetracycline (Table 1).

These results, along with a recent study on an *A. johnsonii* isolate from hospital sewage and also bearing a bla_{OXA-58}-carrying untypable plasmid, add evidence that this species could act as an important carbapenem resistance reservoir [5].

In summary, this work revealed a high diversity of *Acinetobacter* spp. scattered in different settings in this region and the occurrence of CHDL-producing non-*baumannii Acinetobacter* spp. in non-clinical niches. Remarkably, expression of OXA-23 and OXA-58 in these species remained low, which may facilitate their silent dissemination. However, we cannot exclude the development of high levels of carbapenem resistance under antibiotic selective pressure or with genetic transfer to other *Acinetobacter* spp., as we observed with *A. baumannii* transformation with the bla_{OXA-58}-carrying plasmid. Although the origin of the carbapenemases in these particular species remains unclear, there is a possible local enrichment of particular CHDL-producers, alerting for the risk of less explored environmental *Acinetobacter* spp. in other settings worldwide. Moreover, human exposure to CHDL-producing *Acinetobacter* described here, likely associated with ineffective water treatment, is particularly worrisome for vulnerable populations living in Angola.

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Competing interests: None declared.

Ethical approval: Not required.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2016.06.003.

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Supplementary Table S1
Diversity of *Acinetobacter* spp. isolates recovered from different origins in Angola (2013)

Origin/sample identification		Species (no.)	PFGE type [a]	Antibiotic resistance profile	Commune [b]
Healthy volunteers					
Rectal swab	HV 19	*A. baumannii* (1)	**Ab_{I}**	CRO	Benguela
	HV 28	*A. baumannii* (1)	Ab_{II}	CRO	
		A. ursingii (2)	Au_{I} (2)	CRO, FEP	
	HV 29	*A. baumannii* (3)	**Ab_{III}**, Ab_{IV}, **Ab_{V}**	CRO	
		A. baumannii (2)	Ab_{VI} (2)	CRO	
	HV 34	*A. baumannii* (3)	Ab_{VII} (3)	CRO	
		A. ursingii (1)	Au_{II}	CRO	
	HV 35	*A. bereziniae* (1)	NA	CRO	
		A. baumannii (1)	**Ab_{V}**	CRO, TET	
	HV 37	*A. ursingii* (1)	Au_{III}	CRO, CAZ	
	HV 31	*A. junii* (2)	Aj_{I} (2)	–	Baía Farta
Healthy animals and their respective environments					
Farm A	Animal feed/65	*A. pittii* (3)	Ap_{I}, **Ap_{II}**, Ap_{III}	CRO, (CAZ)	Benguela
	Floor, walls/5	*A. gerneri* (1)	NA	CRO, TET	
	Drinking water/52	*A. junii* (1)	Aj_{II}	–	
		A. pittii (2)	Ap_{IV} (2)	CRO	
Farm B	Floor, walls/10	*A. variabilis* (1)	NA	CRO, TET	Benguela
Farm C	Rectal swabs of healthy animals - calf/13	*A. baumannii* (1)	Ab_{VIII}	CRO	Benguela
	Drinking water/40	*A. baumannii* (2)	Ab_{IX} (2)	CRO	
Farm D	Rectal swabs of healthy animals - calf/14	*A. pittii* (1)	Ap_{V}	CRO	Benguela
		A. soli (1)	As_{I}	CRO	
	Rectal swabs of healthy animals - cow/15	*A. soli* (2)	As_{II} (2)	CRO	
	Animal feed/48	*A. baumannii* (1)	Ab_{X}	CRO, AMK, GEN, TOB, TET	
	Drinking water/55	*A. junii* (3)	Aj_{III} (3)	(CRO)	
Wild park	Drinking water/43	*A. baumannii* (2)	**Ab_{XI}**, Ab_{XII}	CRO	Dombe Grande
		A. junii (1)	Aj_{IV}	CRO	
	Drinking water/44	*A. baumannii* (4)	**Ab_{XI}** (4)	CRO	
		A. junii (1)	Aj_{V}	CRO	
	Drinking water/63	*A. pittii* (1)	Ap_{VI}	CRO	
Aquatic environments					
Drinking water for humans	DW/56	*A. baumannii* (1)	Ab_{XIII}	CRO, CAZ	Baía Farta
		A. pittii (3)	**Ap_{VII}** (3)	CRO, (CAZ)	
		A. johnsonii (1)	Ajo_{I}	CRO, CAZ, FEP, TET, CIP	
River (R)	R/38	*A. pittii* (2)	Ap_{VIII}, Ap_{IX}	CRO	Catumbela
	R/39	*A. baumannii* (1)	**Ab_{III}**	CRO	Lobito
		A. johnsonii (2)	Ajo_{II}, Ajo_{III}	(CRO), (FEP)	
		A. junii (2)	Aj_{VI} (2)	(CAZ)	
		A. pittii (1)	Ap_{X}	CRO	
		A. soli (1)	As_{III}	CRO, TET	
		A. towneri-like sp. (1)	At_{I}	IPM, MEM	
	R/42	*A. baumannii* (2)	**Ab_{XI} (2)**	(CRO)	Dombe Grande

Wastewater from a treatment station	Untreated W/45	*A. baumannii* (1)	**Ab_{I}**	CRO	Benguela
		A. junii (1)	Aj_{VII}	CIP	
		A. towneri-like sp. (1)	At_{II}	FEP	
	Treated W/46	*A. junii* (2)	Aj_{VIII}, Aj_{IX}	(CIP)	
Wastewater from an urban sewer line	W/58	*A. pittii* (1)	**Ap_{VII}**	CRO, CAZ	Catumbela
		A. towneri-like sp. (1)	At_{III}	–	
	W/59	*A. pittii* (3)	**Ap_{II}**, Ap_{XI} (2)	CRO	Benguela

PFGE, pulsed-field gel electrophoresis; CRO, ceftriaxone; FEP, cefepime; TET, tetracycline; CAZ, ceftazidime; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; IPM, imipenem; MEM, meropenem; NA, not applicable.

[a] Clonality was established by *Sma*I (for *Acinetobacter junii* and *Acinetobacter johnsonii*) or *Apa*I (for other *Acinetobacter* spp.) pulsed-field gel electrophoresis (PFGE). Pattern analysis was performed by InfoQuest with PFGE types comprising isolates with *Sma*I/*Apa*I patterns presenting similarity levels >74.5%.

[b] Samples were collected from Benguela Province, which comprises nine municipalities (Benguela, Lobito, Bocoio, Balombo, Ganda, Cubal, Caimbambo, Baía Farta and Chongorói), divided in more than 30 communes.

Bold indicates common clones among different samples. Parenthesis indicate variability of a given resistance determinant.

Uncommon carbapenemase-encoding plasmids in the clinically emergent *Acinetobacter pittii*

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Uncommon carbapenemase-encoding plasmids in the clinically emergent *Acinetobacter pittii*

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Objectives: Two carbapenemase-carrying plasmids, pLS488 (bla_{OXA-23}) and pLS535 (bla_{OXA-58}) from *Acinetobacter pittii* clinical isolates, were characterized in this study, including their ability to be transferred to *Acinetobacter baumannii*.

Methods: The clinical isolates were obtained from drainage fluid of a patient with biliary tract cancer and from an exudate of a patient with a hip infection (Portuguese University Hospital, 2012). Isolate characterization included antimicrobial susceptibility tests, carbapenemase production by Blue-Carba, carbapenem-hydrolysing class D β-lactamase (CHDL) gene search by PCR sequencing, ApaI-PFGE, CHDL genetic location and plasmid size by hybridization and WGS. Plasmid transfer was performed by conjugation or electroporation.

Results: pLS488 constitutes the first conjugative plasmid reported to carry a carbapenem resistance gene in *A. pittii* and is part of a potential new incompatibility group that might also account for the dissemination of OXA-23 in *A. baumannii*. pLS535 belongs to the *Acinetobacter* GR7 incompatibility group and presents a new scaffold for OXA-58. This plasmid lacked the machinery for conjugation, but was transferable by electroporation to *A. baumannii*. Both isolates, which displayed the same PFGE pattern, represent the first report of CHDL-carrying *A. pittii* in Portuguese hospitals.

Conclusions: Altogether, these results emphasize the importance of *A. pittii*, or particular *A. pittii* clones, as a source of resistance genes, facilitating their dissemination among different bacterial species.

Introduction

Carbapenem resistance in *Acinetobacter* spp. has been mostly associated with the production of OXA-23, OXA-24/40 and OXA-58 carbapenem-hydrolysing class D β-lactamases (CHDLs).[1,2] The spread of these CHDLs among *Acinetobacter baumannii* has been strongly influenced by clonal expansion, but also by horizontal gene transfer (HGT).[1,3] However, the contribution of HGT to the spread of CHDL among other *Acinetobacter* species, in particular *Acinetobacter pittii*, which is increasingly reported in clinical settings (mainly in Asia, but also in Europe and America, and was recently described in Portugal in lettuce, fruits and raw meat),[2,4,5] has scarcely been explored. To date, only one complete bla_{OXA-58}-carrying plasmid sequence (pOXA58-AP_882) has been described for this species, with no descriptions of bla_{OXA-23}-associated plasmids.[6] In order to obtain new insights into the contribution of *A. pittii* mobile elements to CHDL dissemination, we report here the characterization of two carbapenemase-carrying plasmids, pLS488 (bla_{OXA-23}) and pLS535 (bla_{OXA-58}), exploring at the same time their ability to be transferred to *A. baumannii*.

Materials and methods

Bacterial strains, susceptibility testing and carbapenemase detection

The clinical isolates included in this study were obtained from a Portuguese university hospital that has experienced an endemicity of carbapenemase-producing *A. baumannii* for several years. In more recent years, an increase was observed in the frequency of non-*A. baumannii* species associated with infection and colonization. Thus, following a surveillance screening for carbapenemase producers among non-*A. baumannii* isolates, two *A. pittii* isolates (HGSA488 and HGSA535), collected 3 months apart and presenting decreased susceptibility to carbapenems, were further characterized. HGSA488 was isolated in 2012 from drainage fluid of a patient with biliary tract cancer admitted to the surgery unit, and HGSA535 was isolated from an exudate of a patient with hip infection admitted to the orthopaedic unit. These isolates were initially misidentified as *A. baumannii* by the VITEK 2 system (bioMérieux), but were confirmed as *A. pittii* by MALDI-TOF MS and *rpoB*/*gyrB* partial gene sequencing.[7,8] Antimicrobial susceptibility profiles of different β-lactams, aminoglycosides, tetracyclines, quinolones, trimethoprim/sulfamethoxazole and colistin were obtained by standard disc diffusion, Etest (carbapenems) and broth microdilution (colistin)

methods, according to EUCAST (www.eucast.org) and CLSI guidelines.[9] Carbapenemase production was assessed by the Blue-Carba biochemical assay.[10] CHDL genes were searched for by PCR sequencing, using primers and conditions previously described.[11] Their genetic location and plasmid size were assessed by hybridization of S1 gels with specific probes. Genetic relatedness was characterized by ApaI-PFGE and MLST (Oxford and Pasteur Schemes).

WGS, assembly and plasmid sequence analysis

Total DNA from HGSA488 and HGSA535 was extracted using the Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI) according to the manufacturer's instructions. DNA concentration was estimated using a Qubit dsDNA HS Assay Kit and a Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, USA). Extracted DNA was then sequenced with a standard 2×125 paired-end runs protocol on an Illumina HiSeq 2000 (Illumina, San Diego, CA, USA). The quality of the high-throughput sequence data was assessed by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw sequence reads were then *de novo* assembled using plasmidSPAdes 3.9.0 (http://bioinf.spbau.ru/spades),[12] in order to identify plasmid contigs, and the quality was assessed by QUAST (http://quast.bioinf.spbau.ru). The draft plasmids were automatically annotated with RAST (http://rast.nmpdr.org) and further manually curated by Geneious v. 9.1.6. (Biomatters Limited, Auckland, New Zealand) using BLASTn/BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Conventional PCRs with further Sanger sequencing of amplicons were performed for plasmid circularization [primers pLS488_F (AACCCCACACTACCATCAGC), pLS488_R (CGCTAGGATTCTCTTCAG CTGA), pLS535_F (TGCAAGCATCTACAGTGCCT) and pLS535_R (AGTTGA GGATCAGAACGTAGGG)] and for the detection of the new replicase found in plasmid pLS488 [primers rep_pLS488_F (GTGTTTTGCGGCTTGTTGGA) and rep_pLS488_R (TTGGGTCTTTTTGGCTCGGT)], following the same PCR conditions proposed by Bertini *et al.*[13] Complementary gene identification analysis was done using ISfinder (https://www-is.biotoul.fr), ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) and the Virulence Factors of Bacterial Pathogens database (http://www.mgc.ac.cn/VFs/main.htm).

Mating and electrotransformation experiments

Transfer of plasmids carrying carbapenemase genes was attempted by standard conjugation (filter-mating method at a 1:1 donor/recipient ratio, 37 °C) and electroporation assays, using a rifampicin-resistant mutant of *A. baumannii* ATCC 17978 as a recipient strain. Presumptive transconjugants were selected using Mueller–Hinton II agar plates supplemented with imipenem (0.5 mg/L) and rifampicin (100 mg/L), and presumptive transformants were selected with imipenem (0.5 mg/L). Confirmation was performed with antibiogram and PCR search for carbapenemase genes.

Nucleotide sequence accession numbers

The complete nucleotide sequences of pLS488 and pLS535 plasmids have been submitted to GenBank under accession numbers MF078634 and MF078635, respectively.

Results and discussion

General features of HGSA488 and HGSA535 isolates

HGSA488 displayed an MDR phenotype,[14] being resistant to all β-lactams tested (carbapenems MICs >32 mg/L), except ceftazidime, and susceptible to all non-β-lactams (including colistin, MIC = 0.5 mg/L). This isolate was Blue-Carba positive and presented bla_{OXA-23} associated with plasmid pLS488 (Figure 1). HGSA535 displayed resistance to almost all β-lactams (piperacillin, piperacillin/tazobactam, ticarcillin, amoxicillin/clavulanic acid, aztreonam, cefotaxime and ceftriaxone), except cefepime, ceftazidime, imipenem (MIC = 2.0 mg/L) and meropenem (MIC = 0.75 mg/L), and was susceptible to almost all non-β-lactams (including colistin, MIC = 0.25 mg/L). It is notable that although this isolate was Blue-Carba negative and the imipenem MIC was lower than the clinical resistance breakpoint (4–8 mg/L), it was higher than the epidemiological cut-off value (ECOFF) (1 mg/L), which suggested the acquisition of a carbapenem resistance mechanism, further confirmed by WGS as bla_{OXA-58}, which is associated with plasmid pLS535 (Figure 2). Besides the bla_{OXA-23} and bla_{OXA-58} genes identified, respectively in HGSA488 and HGSA535 isolates, other resistance genes were detected in both genomes, including the natually occurring *A. pittii* β-lactamases $bla_{OXA-500}$ and bla_{ADC-18}.[15] The presence of several genes previously associated with virulence in *A. baumannii* was also observed; these genes were linked to biofilm formation (*adeG*, *csuD*, *bap*), immune evasion (*lpsB*, *lpxA*, *lpxB*, *lpxC*, *lpxD*, *lpxL*), iron uptake systems (*barAB*, *basA*, *basB*, *basD*, *bauA*, *entE*, *hemO*), serum resistance (*pbpG*), outer membrane protein phospholipase (*plcC*) and quorum sensing (*abaI*, *abaR*, *bfmRS*), which can confer an advantage for the maintenance of this lineage in clinical settings. Both isolates belong to ST1030/ST93 (Oxford and Pasteur schemes, respectively) and present the same ApaI-PFGE band pattern (data not shown).

Characterization of plasmid pLS488

The *de novo* assembly of the *A. pittii* HGSA488 produced a single 51316 bp contig, which corresponded to the pLS488 plasmid. This plasmid was further closed by PCR and sequencing of the amplicons, with a final length of 51291 bp, in agreement with the result obtained from S1 nuclease PFGE-based sizing (~51 kb, data not shown). This plasmid comprises 50 ORFs, including genes associated with a transposon structure harbouring bla_{OXA-23} (Tn*2008*) and presenting a GC content of 35.5% (Figure 1), a value compatible with the GC content of *A. pittii* genome.

The plasmid pLS488 backbone was highly related (81% query cover, 99% identity) to a previously described bla_{OXA-23}-harbouring plasmid from an *A. baumannii* strain isolated from the urine of a hospitalized cat with cystitis in Germany (pIHIT7853, GenBank: KX118105.1). The similarity between these plasmids without any known relationship between the isolates suggests that these could be widely disseminated in animals and humans all over the world. On both plasmids, bla_{OXA-23} is embedded in a Tn*2008* transposon, flanked by an *ATPase* gene downstream and a complete IS*Aba1* 27 bp upstream of *bla* [with inverted and double-mutated inverted repeat left (IRL) and inverted repeat right (IRR)]. Tn*2008* was also described in several clinical *A. baumannii* isolates, predominantly in China and the USA, though generally without characterization of the genetic background.[16,17] Curiously, our previous local epidemiology studies[18] showed that OXA-23 has been associated with a different genetic background from the one here described, always being located in transposon Tn*2006* on chromosomes of *A. baumannii* species. Also similar to pIHIT7853, pLS488 displayed a DNA primase (99% identity), involved in DNA replication, the conserved replication module comprising the characteristic *parAB*-partitioning operon and the plasmid replicase [94% identity with the one from pIHIT7853 (accession number KX118105.1)] that ensure the maintenance and inheritance of the plasmid. However,

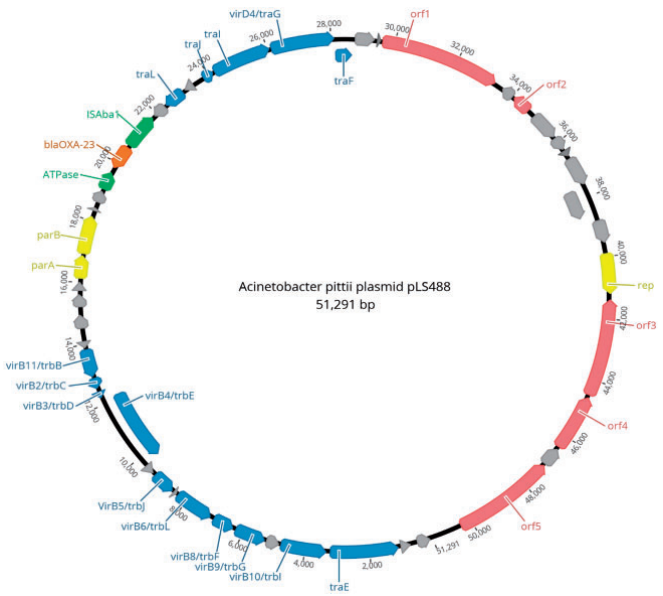

Figure 1. Schematic representation of plasmid pLS488 of *A. pittii* strain HGSA488. Broad arrows in the outer circle indicate genes coding for resistance factors (orange), conjugative transfer genes (blue), replication module (yellow), insertion sequences/mobile elements (green), other factors (red) and hypothetical proteins (grey). *orf1*, DNA primase; *orf2*, resolvase Ser-recombinase superfamily; *orf3*, restriction endonuclease subunit R; *orf4*, DNA methylase; *orf5*, helicase. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

unlike those found in other *Acinetobacter* plasmids (mainly repAci6 plasmids), the replicase (rep) of our plasmid and of pIHIT7853 belong to the superfamily RdgC, a family of DNA-binding proteins that modulate the activity of the RecA recombinase, which is involved in replication and homologous recombination processes. RdgC proteins are restricted to the β- and γ-subsections of *Proteobacteria*, mainly found in *Escherichia coli*, *Salmonella enterica* and *Klebsiella pneumoniae*, but also in *Pseudomonas* spp. These two rep genes may represent a new replicase group (suggesting higher plasmid diversity among *Acinetobacter* species) not included in the *A. baumannii* PCR-based replicon typing scheme proposed by Bertini *et al.*[13] Thus, we designed an additional pair of primers to detect a 482 bp fragment that can simplify the identification of this replicase among other *Acinetobacter* plasmids. pLS488 also contains a helicase, a resolvase and a restriction endonuclease, enzymes not found in pIHIT7853.

Genes of the conjugation system, including the origin of transfer gene (*oriT*), the MOB_P-type relaxase gene (*traI*) and the type IV coupling protein (T4CP) gene (*virD4*/*traG*) for initiation of conjugation, as well as markers of the type IV secretion system (T4SS), including the translocation channel protein genes (*virB3*/*trbD*, *virB6*/*trbL*, *virB8*/*trbF*, *virB9*/*trbG* and *virB10*/*trbI*), the pilus protein genes (*virB2*/*trbC* and *virB5*/*trbJ*) and the ATPase genes (*virB4*/*trbE*, *virB11*/*trbB* and *virD4*/*traG*), were also present, indicating that pLS488 is conjugative. To our knowledge, this is the first conjugative plasmid reported so far to carry a carbapenem resistance gene in *A. pittii*. Consistent with this, attempts to obtain OXA-23-producing *A. baumannii* transconjugants were successful [with co-transferred resistance to all β-lactams (carbapenem MICs >32 mg/L)], emphasizing the importance of *A. pittii* isolates as a source of resistance genes.

Characterization of plasmid pLS535

The *de novo* assembly of the *A. pittii* HGSA535 genome produced a single 11818 bp contig, which corresponded to the pLS535 plasmid. This plasmid was further closed by PCR and sequencing of the amplicons to give a final length of 11388 bp, in agreement with the result obtained from S1 nuclease PFGE-based sizing (~11 kb, data not shown). This plasmid comprises 16 ORFs, presents a GC content of 33.7% (Figure 2) and has substantial nucleotide differences (34%–37% QC, 98%–99% identity) from other bla_{OXA-58}-harbouring plasmids, such as the *A. pittii* plasmid pAG304 (accession number JQ241790.1), previously described in China, but also with plasmids of other *Acinetobacter* species, such as pWA3 (accession number JQ241791.1) and pAba3207a (accession number NZ_CP015365.1) from *A. baumannii* isolates (China and Mexico, respectively) and pXBB1-9 (accession number NZ_CP010351.1) from an *Acinetobacter johnsonii* isolate (China). The common fragments included the transcription regulator *araC1* and the threonine efflux protein *lysE*, and also genes associated with the platform where OXA-58 is embedded: in the right-hand extremity, with a complete copy of IS*Aba3* identified 30 bp downstream of the stop codon of bla_{OXA-58}; and in the left-hand extremity, with an inverted

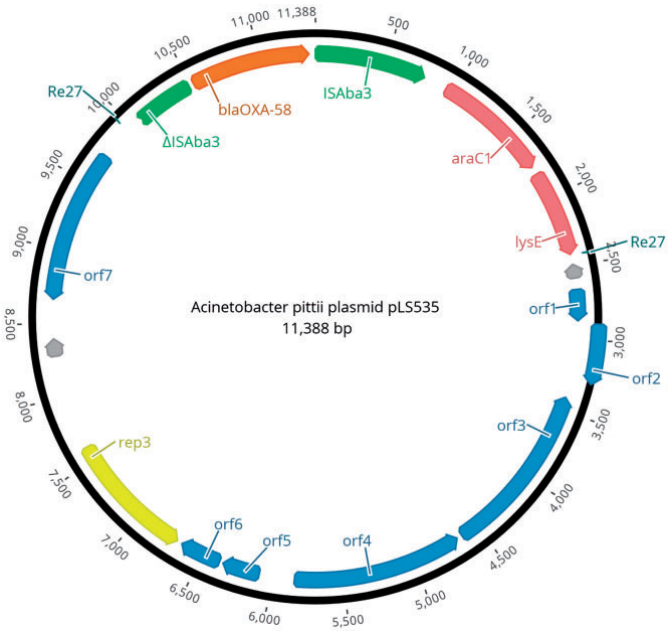

Figure 2. Schematic representation of plasmid pLS535 of *A. pittii* strain HGSA535. Broad arrows in the outer circle indicate genes coding for resistance factors (orange), replication genes (yellow), insertion sequences/mobile elements (green), other factors (red) and hypothetical proteins (grey). Blue arrows indicate putative proteins. *orf1*, putative MazE protein; *orf2*, putative VapC protein; *orf3*, putative Smc protein; *orf4*, putative MobA/MobL; *orf5*, putative mobilization protein; *orf6*, putative DNA-binding protein HU; *orf7*, alcohol dehydrogenase protein. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Δ*ISAba3*, identified 20 bp upstream of the start codon of bla_{OXA-58}. This Δ*ISAba3* was truncated 427 bp after the start codon of the transposase gene of *ISAba3*; this was caused by the C terminus being replaced by the point-mutated IRL of *ISAba32* (found in pD36-4, a *sul2*-harbouring *A. baumannii* plasmid).

Flanking the bla_{OXA-58} locus (downstream of the *lysE* gene and 6 bp upstream of the IRL of *ISAba32*) were two Re27 sequences (ATTTAACATAATGGCGGTTATACGAAG and ATTTAACATAATGGCTGT TATACGAAA), suggesting that a recombination process was responsible for the acquisition of the bla_{OXA-58} locus and might explain the interruption in *ISAba32*. Inside the Δ*ISAba3*, promotor sequences (TTTCTT and TTCTTT) were found, accounting for the expression of OXA-58 and the imipenem MICs being higher than the ECOFF.

On the right-hand end of Re27, a putative VapC–MazE toxin–antitoxin system of the type II module was found.

Even though this plasmid lacked the machinery for conjugation, which explains the failure of the conjugation experiments, we were able to transfer it by electroporation, with *A. baumannii* transformants acquiring resistance to all β-lactams (carbapenems MICs >32 mg/L).

Unlike the bla_{OXA-58}-carrying *A. pittii* plasmids (mainly associated with rep types Aci9, Aci10 and repB)[6,19,20] and the bla_{OXA-58}-carrying *A. baumannii* plasmids from European countries (mostly associated with repAci1),[21] the replicase protein of this plasmid belongs to the Rep3 superfamily (GR7 incompatibility group) and shared 82% nucleotide identity with replicase p3ABSDF0002 from plasmid p3ABSD, as described in France (accession number CU468233.1).

Conclusions

In this study we characterized two new CHDL-carrying *A. pittii* plasmids, pLS488 and pLS535. pLS488 is the first conjugative plasmid associated with a carbapenem resistance gene reported in *A. pittii* and belongs to a potential new incompatibility group, which may also contribute to the dissemination of OXA-23 among *A. baumannii*. pLS535 presents a new scaffold associated with OXA-58. In addition, both isolates represent the first report, to our knowledge, of CHDL-carrying *A. pittii* in Portuguese hospitals. This study also highlights the potential of *A. pittii*, or a particular *A. pittii* clone, to acquire and act as a source of CHDL resistance genes, with the ability to integrate different genetic backgrounds and the potential for further dissemination.

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Transparency declarations

None to declare.

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3.3. Developments on *Acinetobacter* species identification and typing

3.3.1. Discrimination of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex species by Fourier transform infrared spectroscopy.

3.3.2. MALDI-TOF MS and chemometric based identification of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex species.

3.3.3. Development of FTIR-ATR based model for typing clinically-relevant *Acinetobacter baumannii* clones belonging to ST98, ST103, ST208 and ST218.

3.3.4. Unsuitability of MALDI-TOF MS to discriminate *Acinetobacter baumannii* clones under routine experimental conditions.

3.3.5. The secret is on sugar: capsular type explains the discrimination of Acinetobacter baumannii clones by Fourier-transform Infrared (FT-IR) Spectroscopy and Multilocus Sequence Typing.

Discrimination of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex species by Fourier transform infrared spectroscopy

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ARTICLE

Discrimination of the *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex species by Fourier transform infrared spectroscopy

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Abstract The main goal of this work was to assess the ability of Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) to discriminate between the species of the *Acinetobacter calcoaceticus–Acinetobacter baumannii* (Acb) complex, i.e. *A. baumannii*, *A. nosocomialis*, *A. pittii*, *A. calcoaceticus*, genomic species "Between 1 and 3" and genomic species "Close to 13TU". A total of 122 clinical isolates of the Acb complex previously identified by *rpoB* sequencing were studied. FTIR-ATR spectra was analysed by partial least squares discriminant analysis (PLSDA) and the model scores were presented in a dendrogram form. This spectroscopic technique proved to be effective in the discrimination of the Acb complex species, with sensitivities from 90 to 100 %. Moreover, a flowchart aiming to help with species identification was developed and tested with 100 % correct predictions for *A. baumannii*, *A. nosocomialis* and *A. pittii* test isolates. This rapid, low cost and environmentally friendly technique proved to be a reliable alternative for the identification of these closely related *Acinetobacter* species that share many clinical and epidemiological characteristics and are often difficult to distinguish. Its validation towards application on a routine basis could revolutionise high-throughput bacterial identification.

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Introduction

The genus *Acinetobacter* currently comprises 29 distinct species with valid names (http://www.bacterio.cict.fr/a/acinetobacter.html) and a number of taxa which include either genomic species (gen. sp.) delineated by DNA–DNA hybridisation [1] or species with effectively (but not validly) published names. Some of these species are difficult to identify using current methods available in routine clinical laboratories, in particular those belonging to the *Acinetobacter calcoaceticus–Acinetobacter baumannii* (Acb) complex, which encompasses four phenotypically and genotypically related species with valid names, i.e. *A. baumannii*, *A. nosocomialis*, *A. pittii* an *A. calcoaceticus*, and two provisional gen. sp., i.e. the so-called "Between 1 and 3" and "Close to 13TU" [2].

Among the members of the Acb complex, *A. baumannii* is the most clinically important species, which is frequently involved in nosocomial infections including serious outbreaks and associated with increasing reports of multidrug- and pandrug-resistant strains, as well as with higher mortality rates and poorer outcomes when compared with the related species [3, 4].

Moreover, we have assisted to increasing reports of nosocomial infections of non-*A. baumannii* species of the Acb complex [5]. These species differ from *A. baumannii* in characteristics regarding infectious potential, antimicrobial susceptibility and mortality rates, which dictates the need for precise identification to guide the therapy and improve the clinical outcome [4].

A number of genotypic methods have been proposed for *Acinetobacter* species identification [1]. The most widely used identification approaches include polymerase chain reaction (PCR) amplification and sequencing of species-specific DNA regions (e.g. the intrinsic oxacillinases from

different *Acinetobacter* species) [6] or, more recently, partial *rpoB* gene sequence analysis [7]. However, these methods are labourious, time-consuming and expensive. The development of infrared and mass spectrometers such as Fourier transform infrared spectroscopy (FTIR) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) [8] and the availability of sophisticated mathematical tools have lead to a growing interest in the application of spectroscopy in bacterial identification and typing. FTIR combined with multivariate data analysis has already demonstrated an interesting potential for the discrimination of pathogenic and/or Gram-positive and Gram-negative bacteria at different levels (species, subspecies, serotype and, more recently, at the clonal lineage level) [9, 10]. FTIR spectra result from the interaction of infrared radiation with the bacterial isolate, providing a specific fingerprint that reflects the structure and composition of the whole cell [11, 12]. In the attenuated total reflectance (ATR) mode, the infrared beam made contact with the bacterial isolate and became

Table 1 Epidemiologic details of the *Acinetobacter* spp. isolates used in this work

Species	No. of isolates	Sequence type[a]	Country	Year of isolation	Specimen	References
A. baumannii	17	9, 10, 11, 12, 15, 34,35, 36, 37, 38, 39, 40, 47	CZ	1992–2011	Sputum, tracheal secretion, IV cannula, wound, nasal swab, urine, tracheal aspirate	[14, 15]
	2	1	CZ[b]	2011–2012	Sputum, wound	[13]
	7	1, 2, 3, 18, 27, 48, 55	N	1982–2002	Urine, sputum, pharynx, wound, blood	
	1	57	UK	1988	Nailfold	
	1	52	Not known	<1949	Urine	
	37[d]	208[c], 218[c]	P	2010–2012	Urine, bronchial secretion, sputum	[e]
A. nosocomialis	4		CZ	1991–1997	Burn, bronchial secretion, trachea	
	10	68, 71, 76	N	1975–2003	Sputum, urine, blood, autopsy, bronchus, skin, rectum, burn	
	2		S	1980–1981	Gastric fistula, urine	[16]
	1	68	D	1984–1985	Sputum	
	1		C	2001	Skin	
	1		UK	2000	Bronchial secretion	
	1	74	Not known	<1950	Not known	
	1[d]		B	2010	Not known	[e]
A. pittii	6		CZ	1984–2008	Burn, urine	[2]
	9	70, 72, 75	N	1981–1999	Blood, urine, toe web, bronchus	
	1		H	1994	Trachea	
	2	73	S	1980	Wound	[16]
	1	93	D	1993	Axilla	
	1	63	Not known	<1967	Cerebrospinal fluid	
	1[d]		B	2010	Not known	[e]
A. calcoaceticus	2		CZ	1991, 2006	Burn, sputum	
	5	61, 62	N	1900–2006	IV cannula, urinary catheter, eye, amputation stump	
	2	92	S		Wound	[16]
Genomic species "Close to 13TU"	1		CZ	1996	Tracheal secretion	[14]
	2	90, 91	D	1990–1991	Blood, ulcer	[17]
	1		US	1995–2002	Blood	
Genomic species "Between 1 and 3"	2	88, 89	D	1990–1991	Sputum, abscess	[17]

CZ, Czech Republic; N, Netherlands; UK, United Kingdom; S, Sweden; D, Denmark; C, China; H, Hungary; US, United States; B, Brazil; P, Portugal

[a] Sequence type (ST) based on the Institut Pasteur MLST scheme (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html

[b] One isolate imported from Cyprus

[c] ST designation based on the Oxford MLST scheme (http://pubmlst.org/abaumannii/)

[d] Isolates used to test the model

[e] Unpublished data

attenuated depending on the bacteria in contact with the beam. This is a simple, quick, low cost and environmentally friendly technique that can be used in the elucidation of the bacterial chemical features [11, 12].

In this study, we demonstrate that closely related *Acinetobacter* species belonging to the Acb complex can be discriminated by their molecular composition using FTIR-ATR combined with multivariate data analysis. To

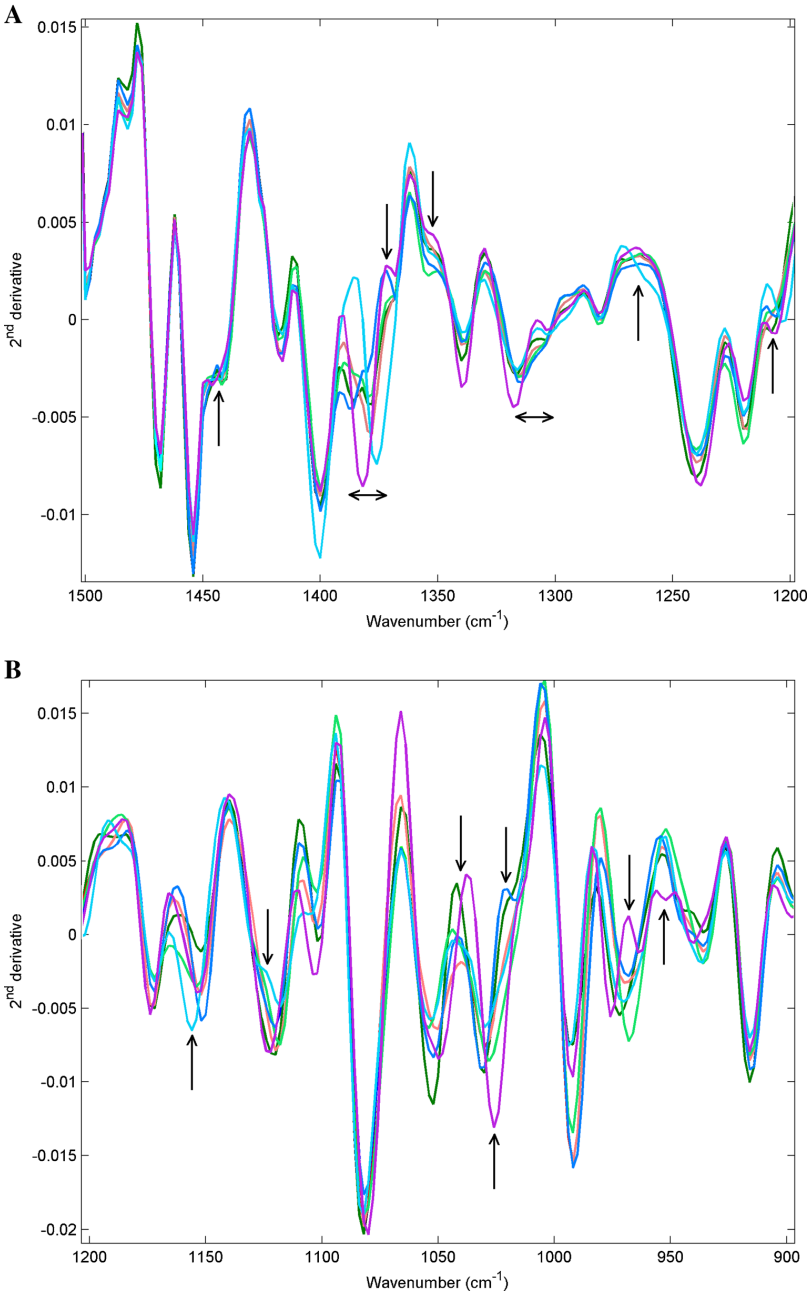

Fig. 1 *Acinetobacter* spp. Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) mean spectra processed with the standard normal variate (SNV) and a Savitzky–Golay filter (seven points filter size, second-degree polynomial, second derivative) in the regions (**A**) 1,500–1,200 cm^{-1} and (**B**) 1,200–900 cm^{-1}. Legend: *A. baumannii*, *A. nosocomialis*, *A. pittii*, *A. calcoaceticus*, gen. sp. "Between 1 and 3", gen. sp. "Close to 13TU". (Colour figure online)

our knowledge, this is the first time that FTIR-ATR spectroscopy is shown to effectively discriminate these relevant species using a large collection of epidemiologically unrelated strains.

Materials and methods

Bacterial isolates

A total of 122 isolates of the Acb complex obtained from human specimens were included in the present study (Table 1). Of these, 83 taxonomically precisely characterised isolates were selected from previous taxonomic and epidemiological studies to reflect the breadth of currently known phenetic diversity within the Acb complex and were used to evaluate the ability of FTIR-ATR in their discrimination: *A. baumannii* (*n*=28), *A. nosocomialis* (*n*=20), *A. pittii* (*n*=20), *A. calcoaceticus* (*n*=9), gen. sp. "Between 1 and 3" (*n*=2) and gen. sp. "Close to 13TU" (*n*=4). An additional 39 test isolates of *A. baumannii* (*n*=37), *A. pittii* (*n*=1) and *A. nosocomialis* (*n*=1) were included to test the reliability of the discrimination. All isolates were identified by *rpoB* sequencing.

FTIR-ATR spectra acquisition

Isolates were grown on Mueller–Hinton agar (37 °C, 18 h) and colonies were directly transferred from the agar plates to the ATR crystal. FTIR-ATR spectra were acquired using a PerkinElmer Spectrum BX FTIR System spectrophotometer with a PIKE Technologies Gladi ATR accessory from 4,000–400 cm^{-1} with a resolution of 4 cm^{-1} and 32 scan co-additions. For each strain, three biological replicates (obtained on three consecutive days) and three instrumental replicates (obtained on the same day from the same agar plate) were obtained and analysed, corresponding to a total of nine spectra for each isolate. Between each isolate measurement, a background was acquired. Due to the large amount of data generated, a mean spectrum (obtained from the nine replicates) was considered in all the analyses.

Spectra pre-processing and modelling

FTIR-ATR spectra were processed with the standard normal variate (SNV) [18], followed by the application of a Savitzky–Golay filter (seven smoothing points, second-order polynomial and second derivative) [19] and mean-centred. Partial least squares discriminant analysis (PLSDA) was the selected supervised method for data analysis [20]. FTIR-ATR spectra were analysed by PLSDA after pre-processing with mean-centring. The PLSDA scores were used as the source for hierarchical cluster analysis (HCA). The purpose of HCA was the generation of dendrograms highlighting the association between isolates. Dendrograms were performed directly on unprocessed PLSDA scores using the Euclidean distance and the Ward's algorithm [18]. All chemometric models were performed in Matlab version 6.5 release 13 (MathWorks, Natick, MA) and the PLS Toolbox version 3.5 for Matlab (Eigenvector Research, Manson, WA).

Results and discussion

Spectral overview

The FTIR-ATR spectra obtained from the 83 isolates studied in this work exhibit the main biomolecules' characteristic infrared absorption bands: lipids (3,000–2,800 cm^{-1}), proteins/amides I and II (1,700–1,500 cm^{-1}), phospholipids/DNA/RNA (1,500–1,185 cm^{-1}), polysaccharides (1,185–900 cm^{-1}) and the fingerprint region (900–600 cm^{-1}) [12]. Unprocessed spectra showed high similarity among the six species (data not shown), with some spectral processing tools being necessary to enlarge spectral variance.

Table 2 Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) peaks identified as biomarkers for the *Acinetobacter calcoaceticus–Acinetobacter baumannii* (Acb) complex species identification

	Peaks (cm^{-1})																	
	Phospholipids/DNA/RNA							Carbohydrates region										
Species	1391	1385	1383	1379	1375	1318	1314	1156	1150	1123	1043	1036	1030	1025	1022	957	953	947
A. baumannii	X			X			X		X		X		X				X	
A. nosocomialis	X			X			X		X		X		X				X	
A. pittii	X			X			X		X		X		X		X		X	
A. calcoaceticus		X			X		X	X		X	X		X				X	
Gen. sp. "Between 1 and 3"	X		X			X			X			X		X		X		X
Gen. sp. "Close to 13TU"	X			X			X		X		X		X		X		X	

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After spectra processing, some differences were observed in the regions of phospholipids/DNA/RNA (Fig. 1a) and carbohydrates (Fig. 1b). These regions were previously used with success in the discrimination of *A. baumannii* [10] and *Streptococcus pneumoniae* [23] and were, therefore, used in the subsequent analysis. The most pronounced spectral differences are marked in Fig. 1a, b with black arrows and summarised in Table 2. Differences in the peak shape observed at 1,445, 1,370, 1,260, 1,220 and 968 cm^{-1}, combined with the presence or absence of specific absorption peaks (Table 1), were helpful in species discrimination.

As seen in Fig. 1a, the band at 1,220 cm^{-1}, usually attributed to the phosphodiester functional groups of DNA/RNA polysaccharide backbone structures, is very similar in *A. baumannii* and *A. nosocomialis*, suggesting a higher degree of similarity concerning the DNA content between these two

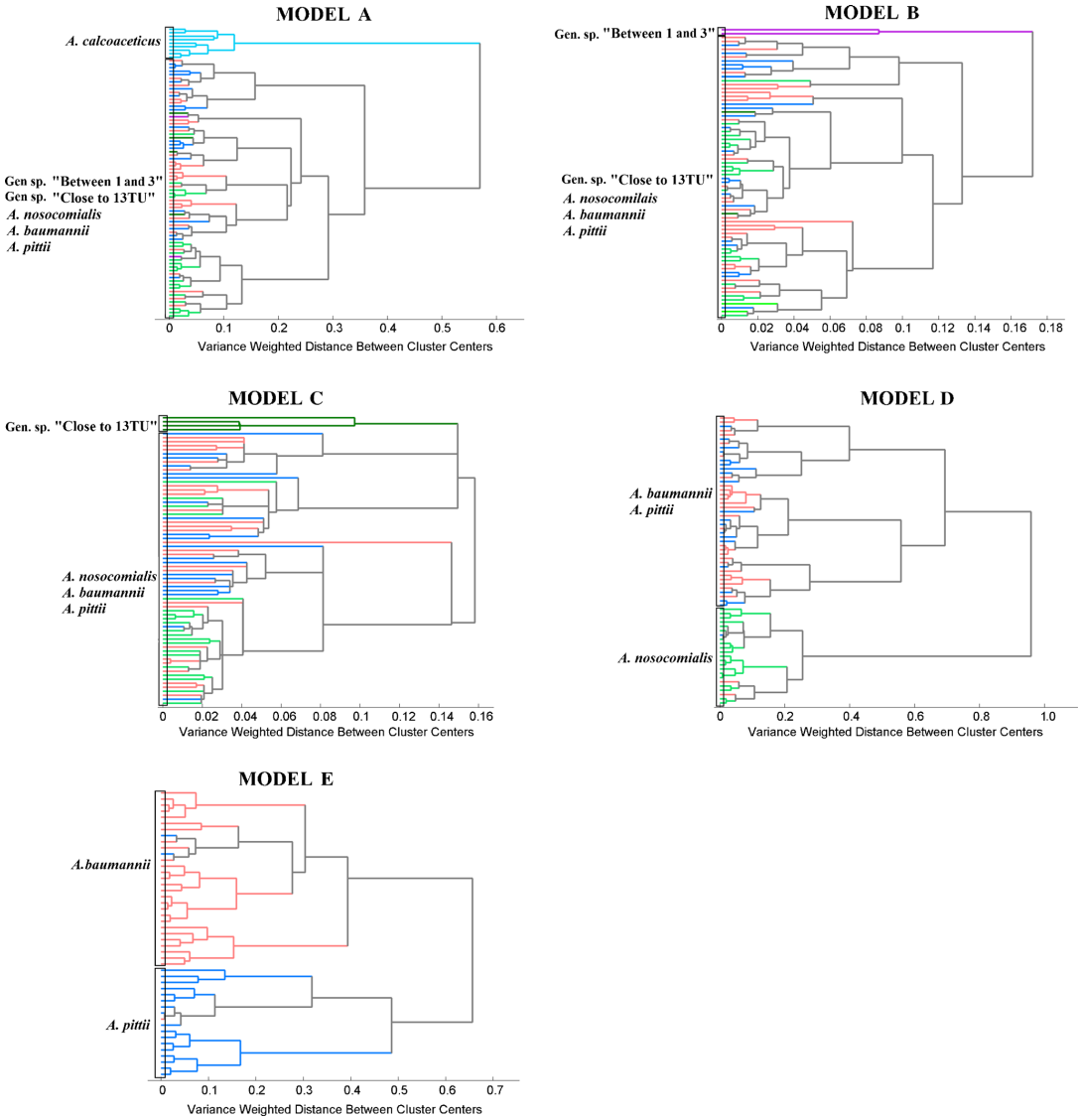

Fig. 2 Dendrograms obtained from the partial least squares discriminant analysis (PLSDA) model scores: models A to E (the agglomerative method used was Ward's algorithm for models A, B, D and E, and the farthest neighbour for model C). Legend: *A. baumannii*, *A. nosocomialis*, *A. pittii*, *A. calcoaceticus*, gen. sp. "Between 1 and 3", gen. sp. "Close to 13TU". (Colour figure online)

species. According Sahl et al. [24], *A. baumannii* and *A. nosocomialis* possess some common genes that are absent in the genomes of the remaining Acb complex species, which is in agreement with the similarity that we found in the DNA content of these two species. The spectra of *A. calcoaceticus* and gen. sp. "Between 1 and 3" were most distant, owing to the presence of specific absorption peaks (Table 1). Spectral differences in the remaining four species were restricted to small peak shifts emphasising the need for multivariate data analysis to better depict spectral species-specific features.

Acinetobacter species discrimination by chemometric methods

Mean spectra were analysed by PLSDA and the model scores were presented in the form of dendrograms, as described previously. The first PLSDA model (Fig. 2, model A), built considering all the isolates belonging to the six species. allowed the discrimination of *A. calcoaceticus* from the other five species in one cluster in the dendrogram. A second PLSDA model (Fig. 2, model B) was then applied to the remaining five species, allowing the discrimination of gen. sp. "Between 1 and 3" in a single cluster. PLSDA models C, D and E (Fig. 2) were consecutively applied and allowed the discrimination of gen. sp. "Close to 13TU", *A. nosocomialis* and *A. pittii* from *A. baumannii*, respectively. In model D, all the *A. nosocomialis* isolates were well predicted. However, the cluster also contains 2/28 *A. baumannii* and 1/20 *A. pittii* isolates, resulting in 100 % sensitivity and 94 % specificity for the discrimination of *A. nosocomialis*. In model E, one *A. baumannii* and two *A. pittii* isolates clustered inaccurately, meaning that the model had 96 % and 90 % sensitivity and 90 % and 96 % specificity for the discrimination of *A. baumannii* and *A. pittii*, respectively. The results herein obtained clearly demonstrate the potential of FTIR-ATR spectroscopy in the identification of closely related species of the *Acinetobacter* genus. Its reliability for routine species identification will be further tested in a greater number of Acb complex isolates.

Flowchart development and test

The results obtained with the different models allowed the establishment of a flowchart aiming to help with species identification (Fig. 3). Spectra of the 39 test isolates were acquired and processed as previously described and projected into the models (models A to E, Fig. 2), according to the established flowchart (Fig. 4). All the test isolates were well assigned to the respective Acb complex species. The robustness of this FTIR-ATR-based method for the identification of the species belonging to the Acb complex is supported by the epidemiological unrelatedness of the test isolates.

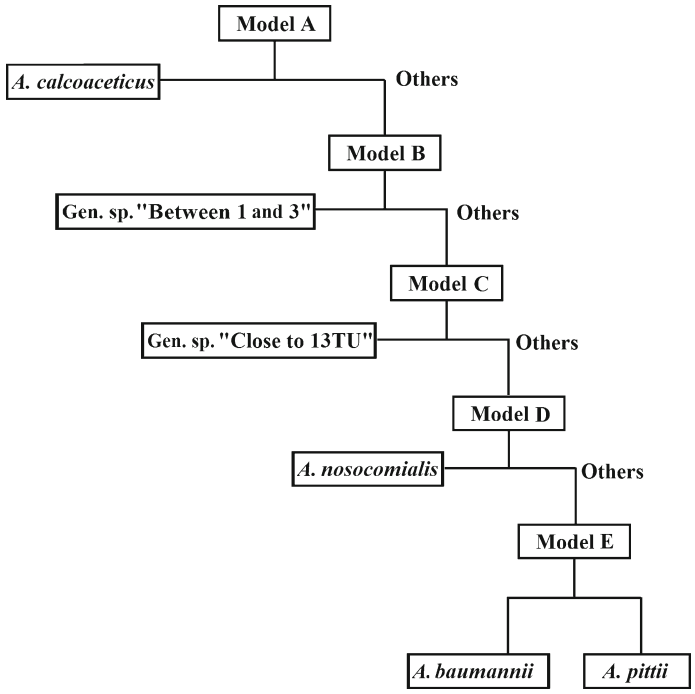

Fig. 3 Flowchart developed for the identification of *Acinetobacter calcoaceticus–Acinetobacter baumannii* (Acb) complex species

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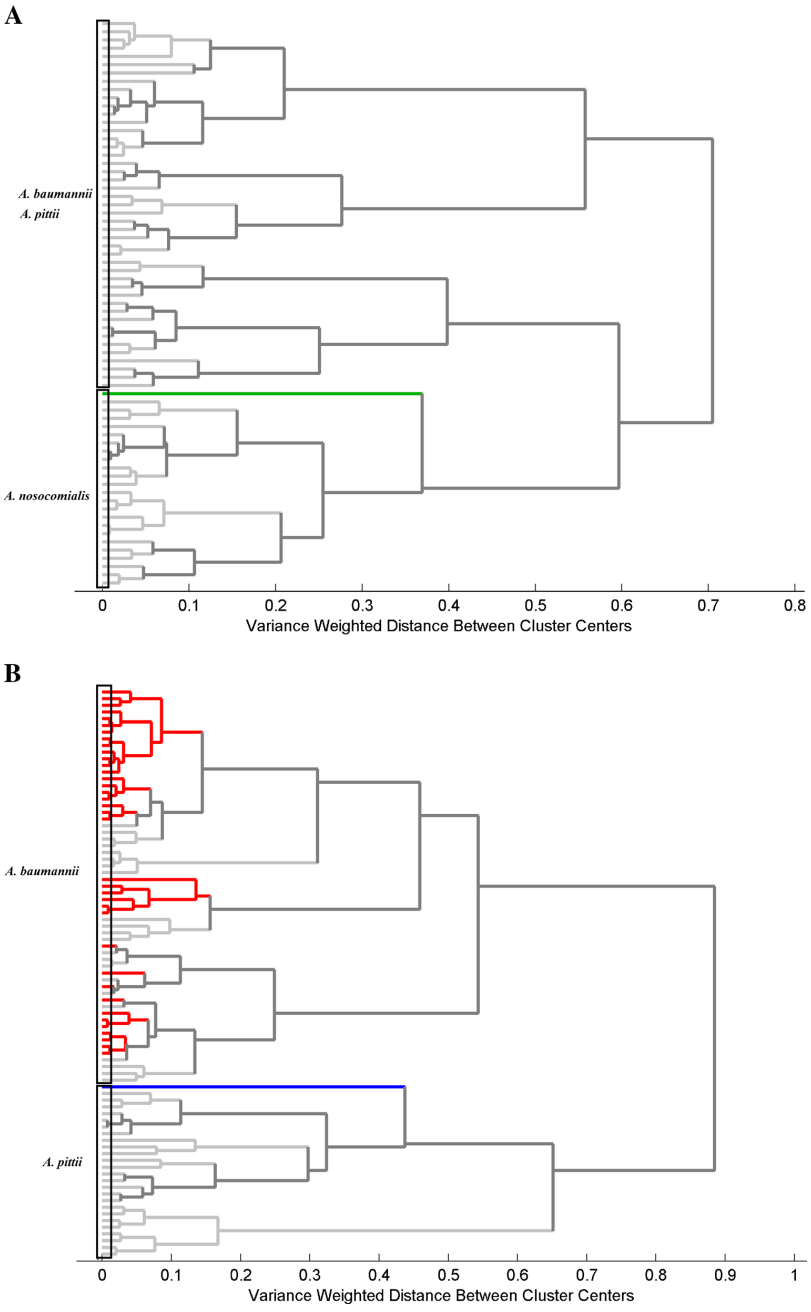

Fig. 4 Test isolates projected into the five developed models according to the flowchart. Legend: (**a**) *A. nosocomialis*, (**b**) *A. baumannii* and *A. pittii*. Isolates used to develop the model are represented in grey (). (Colour figure online)

The high phenetic similarity between the Acb complex species prevents their rapid and reliable identification using routine diagnostic systems [1]. Therefore, time-effective and low-cost techniques are in urgent demand due to the clinical importance of some of these species, mainly *A. baumannii* but also *A. nosocomialis* and *A. pittii* [5]. Despite the potential of FTIR-ATR, its ability to discriminate *Acinetobacter* species was not consistently demonstrated, due to serious flaws in the

bacterial collections tested [21, 22]. In this study, a differential molecular composition among the Acb complex species was depicted through its FTIR-ATR spectra. Using FTIR-ATR spectra coupled with chemometric analysis, the discrimination of closely related *Acinetobacter* species was achieved, and a flowchart was also proposed for the identification of the Acb complex species.

Conclusions

Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) coupled with appropriate chemometric methods demonstrated a reliable ability to identify the closely related species belonging to the *Acinetobacter calcoaceticus–Acinetobacter baumannii* (Acb) complex. The results obtained herein may be of great relevance due to the possibility to identify species with significantly different clinical importance quickly, reliably and with low cost. Furthermore, this study unveils molecular signatures (carbohydrates and phospholipids) of members of the Acb complex that might be further explored to improve the overall knowledge of these bacteria.

Application of fast, reliable and low-cost methods, such as FTIR-ATR, as a diagnostic tool to identify relevant *Acinetobacter* species would significantly impact infection control policies and individual patient management in the clinical context.

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Conflict of interest The authors declare that they have no conflict of interest.

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MALDI-TOF MS and chemometric based identification of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex species

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MALDI-TOF MS and chemometric based identification of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex species

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ABSTRACT

MALDI-TOF MS is becoming the technique of choice for rapid bacterial identification at species level in routine diagnostics. However, some drawbacks concerning the identification of closely related species such as those belonging to the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (Acb) complex lead to high rates of misidentifications. In this work we successfully developed an approach that combines MALDI-TOF MS and chemometric tools to discriminate the six Acb complex species (*A. baumannii*, *Acinetobacter nosocomialis*, *Acinetobacter pittii*, *A. calcoaceticus*, genomic species "Close to 13TU" and genomic species "Between 1 and 3"). Mass spectra of 83 taxonomically well characterized clinical strains, reflecting the breadth of currently known phenetic diversity within the Acb complex, were achieved from intact cells and cell extracts and analyzed with hierarchical cluster analysis (HCA) and partial least squares discriminant analysis (PLSDA). This combined approach lead to 100% of correct species identification using mass spectra obtained from intact cells. Moreover, it was possible to discriminate two Acb complex species (genomic species "Close to 13TU" and genomic species "Between 1 and 3") not included in the MALDI Biotyper database.

Introduction

Mass spectrometry was proposed for bacterial identification about 35 years ago, although with severe confines namely due to the limitations in the ionization of large biomolecules (Anhalt and Fenselau, 1975). The possibility of surpassing this issue with the new MALDI-TOF MS (Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) apparatus made this technique very desirable for clinical routine offering the possibility of rapid and inexpensive identification of bacteria at the species level (Karas et al., 1985; Heller et al., 1987). In recent years, we assisted to an impressive increasing number of studies aiming the evaluation of this technique as a reliable tool for bacterial discrimination, including of closely related species (Pribil and Fenselau, 2005; Carbonnelle et al., 2010; Croxatto et al., 2012). *Acinetobacter* genus has been the target of some of these studies (Espinal et al., 2011; Álvarez-Buylla et al., 2012) due to the clinical relevance of some of its species and the frequent misidentifications namely among the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (Acb) complex species (Nemec et al., 2011). *A. baumannii* is recognized as the most clinically relevant species, being involved in severe nosocomial infections (Park et al., 2013), frequently demonstrating multidrug resistance (Lim et al., 2007; Grosso et al., 2010) and associated with high mortality rates (Park et al., 2013). More recently, non-*A. baumannii Acinetobacter* infections, also associated to resistant strains and with different clinical outcomes (Lai et al., 2012), have been increasingly reported highlighting the need for accurate identification of *Acinetobacter* species.

Previous attempts made with MALDI-TOF MS aiming species identification relied on direct comparisons with MALDI Biotyper database or in small alterations to the procedure recommended by the manufacturer concerning cell preparation (Espinal et al., 2011; Álvarez-Buylla et al., 2012; Mencacci et al., 2013). In this study, we evaluated the potential of MALDI-TOF MS coupled with chemometric tools (PLSDA – Partial Least Squares Discriminat Analysis and HCA – Hierarchical Cluster Analysis) to discriminate the Acb

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complex species (*A. baumannii*, *A. nosocomialis*, *A. pittii*, *A. calcoaceticus*, genomic species (gen. sp.) "Close to 13TU" and gen. sp. "Between 1 and 3"), considering both intact and cell extracts (IC and CE). The results obtained herein clearly show that a combined MALDI-TOF MS and chemometric approach highly improve the rate of correct identifications among closely related species as those belonging to the Acb complex.

Material and methods

Bacterial strains

The study collection included 83 unrelated and taxonomically well characterized strains obtained from clinical specimens in different countries and periods of time and belongs to diverse sequence types in order to provide maximal diversity. The strains were selected from published studies (Nemec et al., 1999, 2004, 2011; Krizova et al., 2012) to reflect the breadth of currently known phenetic diversity within the Acb complex: *A. baumannii* ($n = 28$), *A. nosocomialis* ($n = 20$), *A. pittii* ($n = 20$), *A. calcoaceticus* ($n = 9$), gen. sp. "Close to 13TU" ($n = 4$) and gen. sp. "Between 1 and 3" ($n = 2$). The strains were previously identified at the species level by *rpoB* sequencing and most of them were also characterized by other taxonomic methods such as multilocus sequence typing (MLST) or AFLP whole-genome fingerprinting (Nemec et al., 1999, 2004, 2011; Krizova et al., 2012). In addition, all the strains were distinct at the strain level as evidenced by several genotyping methods. Details about the strains included in this study are summarized in Tables 1 and 2.

MALDI-TOF MS

MALDI-TOF MS experiments were performed directly with intact cells and with cell extracts for comparison. Intact cells were obtained from an overnight culture in Mueller-Hinton agar (37 °C/18 h). Cell extracts were prepared according the manufacturer instructions. Briefly, overnight cultures in Muller-Hinton agar were suspended in HPLC water and treated with ethanol (75%). After centrifugation and removal of the supernatant, cells were extracted with 25 μL of 70% formic acid followed by addition of 25 μL of acetonitrile and vortexing at 2000 rpm for 1 min. Samples, both intact bacterial cells and cell extracts, were spotted onto MALDI ground steel target (AnchorChip) followed by drying and the addition of 1 μL of the chemical matrix (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% of acetonitrile and 2.5% of trifluoroacetic acid). Spectra were randomly obtained from blind samples in the linear positive mode at a laser (nitrogen) frequency of 20 Hz in the range of 2–20 kDa with a Microflex III instrument (Bruker Daltonic, Bremen, Germany). Each recorded spectrum is the results of six series of 40 single laser shots in different locations. The experiments were performed in quadruplicate using four distinct spots of the MALDI target (instrumental replicates) at least in two different days (biological replicates) with two different bacterial cultures or extracts. External calibration of the mass spectra was performed using *Escherichia coli* DH5 alpha standard peaks (BTS).

MALDI-TOF peaks were directly compared with the reference spectra of the integrated database using MALDI Biotyper Software (MALDI Biotyper Version 3.0 Build 25 – Bruker Daltonics) and subjected to chemometric analysis.

Chemometric analysis

Due to the large amount of data generated by MALDI experiments, mean spectra for each isolate were generated from the instrumental and biological replicates and considered for further analysis. Mass spectra were previously analyzed with the *peakfind* function of the PLS Toolbox for Matlab (settings: 9 points for the Savitzky–Golay filter, 6 units for the peak tolerance and 19 points to estimate local maxima) to evaluate the intra and interspecies variability among the six species. Additionally, for clustering purposes spectra were analyzed by partial least squares discriminant analysis (PLSDA) and hierarchical cluster analysis (HCA) (Barker and Rayens, 2003; Sousa et al., 2014) after pre-processing with mean-centring. In PLSDA, to each known sample (x_i) is assigned a vector of zeros with the value one at the position corresponding to its species (y_i). The structure of the PLSDA model is described by Eqs. (1) and (2). Model loadings (P and Q) and corresponding scores (T and U) are obtained by sequentially extracting the components or latent variables (LV) from matrices X (the spectra) and Y (the matrix codifying the STs).

$$X = TP^t + E \tag{1}$$

$$Y = UQ^t + F \tag{2}$$

The algorithm correlates the scores of each block (T and U), yielding an internal regression matrix. This internal regression can be transformed on a regression matrix (B). In this case, the regression matrix is composed by three vectors: one regression vector corresponding to each ST. E and F are the residual matrices and depend on the number of latent variables selected. Predictions for new samples are obtained by multiplying a new spectrum (x_{new}) by the regression matrix (B).

$$y_{new} = x_{new}B \tag{3}$$

The prediction ($y_{new} = [y_{new,1}, y_{new,2}, \ldots, y_{new,n}]$) is then converted in a class assignment. In PLSDA a probability value for each assignment is estimated for each sample. The model was calibrated and tested with the leave-one-sample-out cross-validation procedure in order to prevent overfitting. The cross validation (where 30% of randomly selected samples are used to test the model) was performed 100 times. The PLSDA scores were used as the source for the HCA. The purpose of HCA was the generation of dendrograms highlighting the association between strains. Dendrograms were generated directly on unprocessed PLSDA scores using the k-nearest neighbor agglomerative method (Næs et al., 2002).

Sensitivity and specific values of the chemometric approach were obtained through a cross validation strategy of the PLSDA model (Sousa et al., 2014) where 70% of the strains where randomly selected to calibrate the model and 30% to test the model (the procedure was repeated 100 times).

All chemometric models were performed in Matlab version 7.4 Release 2007a (MathWorks, Natick, MA) and PLS Toolbox version 4.2.1 for Matlab (Eigenvector Research, Manson, WA).

Results

Species identification with the integrated database of the MALDI Biotyper software

Species identification through direct spectra comparison with the integrated Biotyper database revealed 34 *A. baumannii*, 18 *A. nosocomialis*, 21 *A. pittii* and 10 *A. calcoaceticus* using IC and 36 *A. baumannii*, 17 *A. nosocomialis*, 20 *A. pittii* and 10 *A. calcoaceticus* with CE (Table 3). It is of note that none of the species was identified with mean scores greater than 2.300 meaning, according to the manufacturer, that only a probable identification at the species level can be achieved using the integrated database of the MALDI Biotyper Software.

Considering IC, all *A. baumannii*, *A. pittii* and *A. calcoaceticus* strains were accurately identified. Nevertheless, two strains of *A. nosocomialis* (2/20) and four strains of gen. sp. "Close to 13TU" (4/4) were identified as *A. baumannii* and the two strains of gen.

Table 1
Epidemiologic characteristics of the strains studied.

Acinetobacter species	Strain no.	Human specimen	Country of isolation	Year of isolation	ST[a]	Note	Ref
A. baumannii	NIPH 60	Sputum	CZ	1992	34		Nemec et al. (2011)
	NIPH 67	Tracheal secretion	CZ	1992	35		Nemec et al. (2011)
	NIPH 70	Tracheal secretion	CZ	1992	36		Nemec et al. (2011)
	NIPH 80	IV cannula	CZ	1993	37		Nemec et al. (2011)
	RUH 134 (=NIPH 528)	Urine	NL	1982	2	EU clone II reference strain	Nemec et al. (2011)
	NIPH 146	Wound	CZ	1993	25		Nemec et al. (2011)
	NIPH 190	Tracheal secretion	CZ	1993	9		Nemec et al. (2011)
	NIPH 201	Nasal swab	CZ	1992	38		Nemec et al. (2011)
	NIPH 290	Urine	CZ	1994	1	EU clone I	Nemec et al. (2011)
	NIPH 329	Tracheal secretion	CZ	1994	11		Nemec et al. (2011)
	NIPH 335	Sputum	CZ	1994	10		Nemec et al. (2011)
	NIPH 410	Cannula	CZ	1996	39		Nemec et al. (2011)
	NIPH 501[T]	Urine	Not known	<1949	52	Type strain	Nemec et al. (2011)
	RUH 875 (=NIPH 527)	Urine	NL	1984	1	EU clone I reference strain	Nemec et al. (2011)
	NIPH 601	Urine	CZ	1993	40		Nemec et al. (2011)
	NIPH 615	Tracheal secretion	CZ	1994	12		Nemec et al. (2011)
	NIPH 1362	Tracheal aspirate	CZ	2000	47	EU clone II	Nemec et al., 2004
	NIPH 1734	Sputum	CZ	2001	15		Nemec et al. (2011)
	NIPH 2061	IV cannula	CZ	2003	2	EU clone II	Périchon et al. (2014)
	RUH 2180	Sputum	NL	1987	27		Nemec et al. (2011)
	RUH 2688	Pharynx	NL	1987	55		Nemec et al. (2011)
	RUH 3414	Nailfold	UK	1988	57		Nemec et al. (2011)
	LUH 8088	Sputum	NL	2002	48		Nemec et al. (2011)
	LUH 8326	Wound	NL	2002	18		Nemec et al. (2011)
	ANC 4097	Sputum	CZ (import from Egypt)	2011	1	EU clone I	Krizova et al. (2012)
	ANC 4201	Sputum	CZ	2011	2	EU clone II	
	ANC 4373	Wound swab	CZ (import from Cyprus)	2012	N.D.	EU clone I	
	NIPH 1669 (=LUH 5875)	Blood	NL	1997	3	EU clone III reference strain	Nemec et al. (2011)
A. nosocomialis	NIPH 12	Burn	CZ	1991	N.D.		Nemec et al. (2011)
	NIPH 97	Bronchial secretion	CZ	1993	N.D.		Nemec et al. (2011)
	NIPH 106	Tracheal secretion	CZ	1993	N.D.		Nemec et al. (2011)
	NIPH 386	Sputum	CZ	1996	410		Nemec et al. (2011)
	NIPH 523 (=LMG 993)	Not known	Not known	<1950	74		Nemec et al. (2011)
	NIPH 2119[T]	Sputum	NL	1987	76	Type strain	Nemec et al. (2011)
	RUH 503 (=LMG 10,620)	Urine	NL	1984	68		Nemec et al. (2011)
	RUH 412 (=LMG 10,624)	Blood	NL	1975–1980	N.D.		Nemec et al. (2011)
	RUH 2041 (=LMG 10,621)	Autopsy	NL	1986	N.D.		Nemec et al. (2011)
	RUH 2211 (=LMG 10,625)	Gastric fistula	SE	1980–1981	N.D.		Nemec et al. (2011)
	RUH 2212 (=LMG 10,626)	Urine	SE	1980–1981	N.D.		Nemec et al. (2011)
	RUH 2284 (=LMG 10,622)	Bronchus	NL	1987	N.D.		Nemec et al. (2011)
	RUH 2624 (=LMG 10,617)	Skin	NL	1987	71		Nemec et al. (2011)
	RUH 2627 (=LMG 10,623)	Rectum	NL	1987	N.D.		Nemec et al. (2011)
	RUH 3417	Sputum	DE	1984–1985	68		Nemec et al. (2011)
	LUH 7715	Sputum	NL	2000	71		Nemec et al. (2011)
	LUH 6585	Burn	NL	2001	N.D.		Nemec et al. (2011)
	LUH 7430	Skin	CN	2001	N.D.		Nemec et al. (2011)
	LUH 8732	Sputum	NL	2003	N.D.		Nemec et al. (2011)
	LUH 7150	Bronchial secretion	UK	2000	N.D.		Nemec et al. (2011)

Table 1 (*Continued*)

Acinetobacter species	Strain no.	Human specimen	Country of isolation	Year of isolation	ST[a]	Note	Ref
A. pittii	NIPH 14	Burn	CZ	1991	N.D.		Nemec et al. (2011)
	RUH 37 (=LMG 10,560)	Blood	NL	1981	N.D.		Nemec et al. (2011)
	NIPH 76	Urine	CZ	1992	N.D.		Nemec et al. (2011)
	NIPH 95	Urine	CZ	1993	N.D.		Nemec et al. (2011)
	NIPH 336	Urine	CR	1993	N.D.		Nemec et al. (2011)
	RUH 468 (=LMG 10,562)	Urine	NL	1984	N.D.		Nemec et al. (2011)
	NIPH 519[T]	Cerebrospinal fluid	Not known	<1967	63	Type strain	Nemec et al. (2011)
	NIPH 789	Trachea	HU	1994	N.D.		Nemec et al. (2011)
	RUH 1163 (=LMG 10,555)	Toe web	NL	1985	75		Nemec et al. (2011)
	RUH 509 (=LMG 10,559)	Bronchus	NL	1984	72		Nemec et al. (2011)
	RUH 502 (=LMG 10,554)	Drain	NL	1984	N.D.		Nemec et al. (2011)
	RUH 408 (=LMG 10,561)	Not known	CH	1984	N.D.		Nemec et al. (2011)
	RUH 532 (=LMG 10,564)	Urine	NL	1984	N.D.		Nemec et al. (2011)
	RUH 1020 (=LMG 10,557)	Bronchus	NL	1984	N.D.		Nemec et al. (2011)
	RUH 2205 (=LMG 10,556)	Wound	SE	1980	N.D.		Nemec et al. (2011)
	RUH 2204 (=LMG 10,553)	Wound	SE	1980	73		Nemec et al. (2011)
	RUH 1944	Urine	NL	1986	70		Nemec et al. (2011)
	ANC 3678	Water with mud	CZ	2008	N.D.		Nemec et al. (2011)
	LUH 5968	Urine	NL	1999	N.D.		Nemec et al. (2011)
	ANC 3870 (=SH024)	Axilla	DE	1993	93		Nemec et al. (2011)
A. calcoaceticus	NIPH 13 (=CCM 4665)	Burn	CZ	1991	N.D.		Nemec et al. (2011)
	RUH 582 (=LMG 10,516)	Soil	NL	1984	N.D.		Nemec et al. (2011)
	RUH 944 (=LMG 10,515)	IV cannula	NL	1984	N.D.		Nemec et al. (2011)
	RUH 2202 (=LMG 10,517)	Wound	SE	1980–1981	92		Nemec et al. (2011)
	RUH 2203 (=LMG 10,518)	Wound	SE	1980–1981	N.D.		Nemec et al. (2011)
	NIPH 2706	Sputum	CZ	2006	N.D.		Nemec et al. (2011)
	LUH 9144	Urinary catheter	NL	2004	61		Nemec et al. (2011)
	LUH 11,899	Eye	N	2006	N.D.		Nemec et al. (2011)
	LUH 2005	Amputation stump	N	1994	N.D.		Nemec et al. (2011)
Genomic sp. 'Close to 13TU'	NIPH 417	Tracheal secretion	CZ	1996	N.D.		Nemec et al. (1999)
	NIPH 826 (=LUH 1471)	Blood	DK	1990–1991	90		Nemec et al. (2011)
	NIPH 973 (=LUH 1472)	Ulcer	DK	1990–1991	91	Reference strain of 'Close to 13TU'	Nemec et al. (2011)
	ANC 4045	Blood (human)	US	1995–2002	N.D.		–
Genomic sp. "Between 1 & 3"	NIPH 542 (=LUH 1470)	Sputum	DK	1990–1991	89		Nemec et al. (2011)
	NIPH 817 (=LUH 1469)	Abscess	DK	1990–1991	88		Nemec et al. (2011)

CZ, Czech Republic; NL, Netherlands; UK, United Kingdom; SE, Sweden; DK, Denmark; CN, China; HU, Hungary; US, United States of America, CH, Switzerland, DE, Germany. N.D., not determined.

[a] MLST scheme of Diancourt et al. (2010) (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html).

Table 2
The *rpoB* gene similarity values within and between the species of the ACB complex.[a]

Species	*A. baumannii*	*A. nosocomialis*	Genomic sp. 'Close to 13TU'	*A. pittii*	*A. calcoaceticus*	Genomic sp. "Between 1 and 3"
A. baumannii	98.7–100					
A. nosocomialis	94.2–95.4	98.7–100				
Genomic sp. 'Close to 13TU'	94.3–94.9	94.9–95.6	99.2–100			
A. pittii	92.5–93.5	92.7–93.4	91.6–92.3	99.0–100		
A. calcoaceticus	90.9–92.2	90.2–91.8	90.4–91.6	92.3–93.7	98.1–100	
Genomic sp. "Between 1 and 3"	91.0–91.8	91.1–91.8	90.8–91.2	92.3–92.9	96.8–97.9	99.3

[a] Calculations are based on the 861 bp region encompassing the nucleotide positions 2915–3775 of the *rpoB* coding region. Pairwise similarity values are expressed as the percentages of identical nucleotides in the corresponding positions of two aligned sequences.

sp. "Between 1 and 3" strains were misidentified (2/2), one as *A. pittii* and one as *A. calcoaceticus*.

In the case of CE, only *A. baumannii* and *A. calcoaceticus* strains were correctly identified. Four strains of *A. nosocomialis* (4/20) and four of gen. sp. "Close to 13TU" (4/4) were misidentified as *A. baumannii*; one strain of *A. pittii* (1/20) was misidentified as *A. nosocomialis* and two strains of gen. sp. "Between 1 and 3" were misidentified (2/2) one as *A. pittii* and one as *A. calcoaceticus*.

It is of note that higher sensitivity and specificity were obtained using IC as compared to CE, for species identification through the MALDI Biotyper database (Table 4).

Spectral overview

Fig. 1(A (IC) and B (CE)) summarizes the position of the peaks found with *peakfind* function for each strain. The position of the peaks was achieved from the mean mass spectra for each strain, obtained as described previously, and no intra-strain variability was observed (data not shown). Considering IC, species mass peaks were mainly concentrated in the region below 10,000 *m*/*z* (about 99%) and almost no peaks could be found above 13,000 *m*/*z*. Some degree of spectral variability was also observable within each species (no more than 5%); however a consistent species pattern was notorious. Mass spectra of the CE lead to a major number of peaks than IC mainly in the region above 10,000 *m*/*z* and higher intra-species variability (about 5–10%). Nevertheless, a species peak pattern was also seen.

A detailed spectra analysis of each species was also performed in the region between 3000 and 10,000 *m*/*z* due to its higher inter-species variability, Fig. 2(A (IC) and B (CE)). Peaks at 4662, 5176, 6949, 7435 and 9323 *m*/*z*, present in both IC and CE (only with small shifts), were found to be common in all the species being probably associated with the *Acinetobacter* genus. Moreover, all the species seems to have at least one specific peak found with IC and CE (*A. baumannii*: 5749 *m*/*z*; *A. nosocomialis*: 8139 *m*/*z*; *A. pittii*: 5779 *m*/*z*; *A. calcoaceticus*: 5833 *m*/*z*; gen. sp. "Close to 13 TU": 9542 *m*/*z*; gen. sp. "Between 1 & 3": 3908 *m*/*z*).

Chemometric analysis

Dendrograms obtained for IC and CE were generated with 4 and 8 latent variables encompassing 70.4% and 80.4% of the spectral variability, respectively.

Considering IC, the resulting dendrogram (Fig. 3A) exhibits six perfectly defined clusters each comprising strains of a single species and in accordance with their identification by *rpoB*. The cross validation procedure showed 89% of sensitivity for *A. calcoaceticus* and 100% for the remaining 5 species and 95% of specificity for *A. nosocomialis* and 100% for the other species (Table 4). Concerning CE the discrimination of the six species is unfeasible with results of the cross validation for sensitivity from 75% to 100% and for specificity lower than 100% except for *A. pittii* (Table 4). The dendrogram (Fig. 3B) showed three well defined clusters corresponding to gen. sp. "Between 1 and 3", gen. sp. "Close to 13TU" and *A. pitti* strains. However some degree of overlapping was observed between the clusters containing *A. calcoaceticus*, *A. baumannii* and *A. nosocomialis* being the discrimination of these 3 species unachievable with CE.

Discussion

In this work, we developed a combined MALDI-TOF MS and chemometrics approach to accurately discriminate six closely related species belonging to the Acb complex.

Several recent studies (Böhme et al., 2010; Espinal et al., 2011; Álvarez-Buylla et al., 2012) have addressed the potential of this high throughput technique for *Acinetobacter* species identification; however always through direct MALDI Biotyper database

Table 3
Acinetobacter species identification and mean score values obtained through intact cell (IC) and cell extract (CE) direct mass spectra comparison with MALDI Biotyper database.

Species[a] (n° strains)	IC identification		CE identification	
	Species (n° strains)	Score[b]	Species (n° strains)	Score[b]
A. baumannii (28)	*A. baumannii* (28)	2–2.3	*A. baumannii* (28)	2–2.3
A. nosocomialis (20)	*A. nosocomialis* (18)	2–2.3	*A. nosocomialis* (16)	2–2.3
	A. baumannii (2)	2.095	*A. baumannii* (4)	1.901
A. pittii (20)	*A. pittii* (20) 2–2.3		*A. pittii* (19)	2–2.3
			A. nosocomialis (1)	1.981
A. calcoaceticus (9)	*A. calcoaceticus* (9)	2–2.3	*A. calcoaceticus* (9)	2–2.3
Gen. sp. "Close to 13TU" (4)	*A. baumannii* (4)	2–2.3	*A. baumannii* (4)	2–2.3
Gen. sp. "Between 1 and 3" (2)	*A. pittii* (1)	1.878	*A. pittii* (1)	1.816
	A. calcoaceticus (1)	1.797	*A. calcoaceticus* (1)	1.855

[a] *rpoB* based identification.
[b] Results were expressed as log (score) values in the range of 0–3. Scores between 2.300 and 3.000 indicate a trustworthy identification at species level, between 2.000 and 2.299 trustworthy at genus level and probable at species level, between 1.700 and 1.999 probable at genus level and lower than 1.699 no identification can be made.

Table 4
Specificity and sensitivity of Acb complex species identifications obtained through direct database comparison and chemometric analysis, of intact cells (IC) and cell extracts (CE) mass spectra.

Species	Direct database comparison				Chemometric methods[a]			
	Sensitivity (%)		Specificity (%)		Sensitivity (%)		Specificity (%)	
	IC	CE	IC	CE	IC	CE	IC	CE
A. baumannii	100	100	89	85	100	96	100	96
A. nosocomialis	90	80	100	98	100	100	95	97
A. pittii	100	95	98	98	100	100	100	100
A. calcoaceticus	100	100	99	99	89	100	100	99
Gen. sp. "Close to 13TU"	N.A.	N.A.	N.A.	N.A.	100	75	100	98
Gen. sp. "Between 1 and 3"	N.A.	N.A.	N.A.	N.A.	100	100	100	98

Sensitivity – proportion (or percentage) of strains correctly identified at the species level; *Specificity* – proportion (or percentage) of all the strains that are correctly identified as not belonging to a given species. N.A. – not applicable.
[a] Values related to the cross validation method leave-one-out.

comparisons and reporting a high rate of species misidentifications. The results obtained in our work lead to a higher percentage of correct species identifications through the MALDI Biotyper database (90% considering IC and 87% considering CE) than previous studies (Böhme et al., 2010; Espinal et al., 2011) which possibly result from database improvements, namely more reference profiles of *A. baumannii* ($n = 5$) and *A. calcoaceticus* ($n = 1$) were added and *A. nosocomialis* ($n = 5$) profiles were newly included.

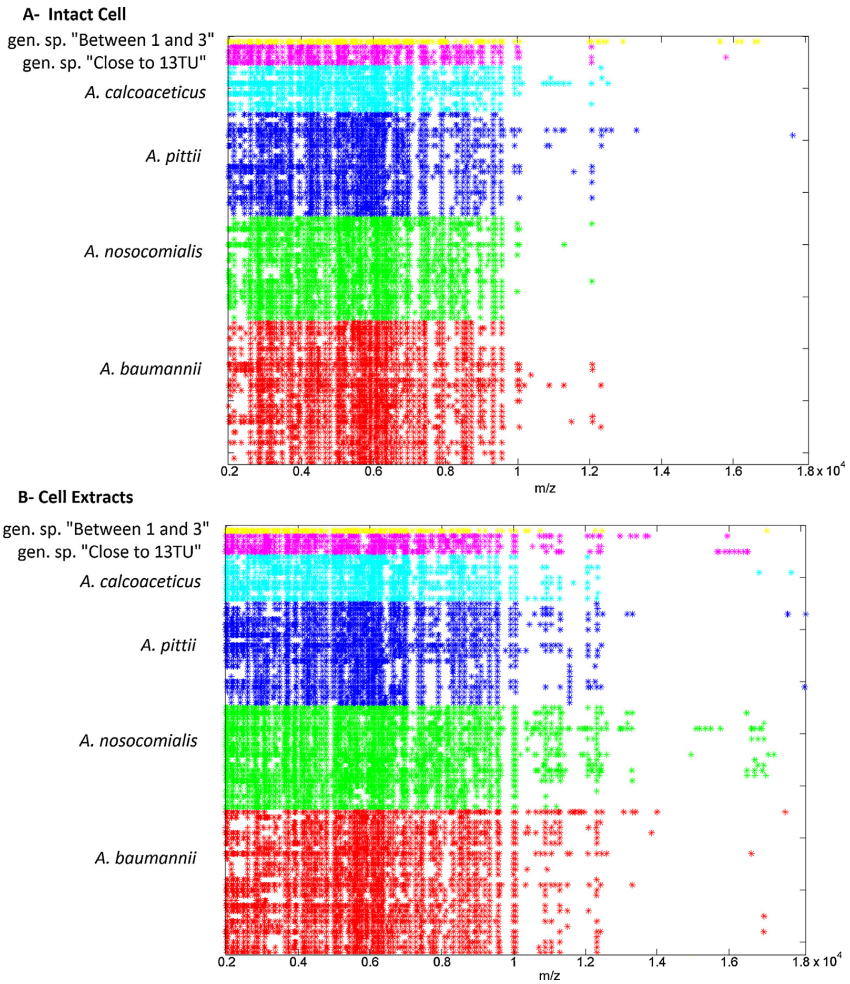

Fig. 1. Peak positions (m/z) of the 83 strains obtained with IC (A) and CE (B). Legend: ✱, *A. baumannii*; ✱, *A. nosocomialis*; ✱, *A. pittii*; ✱, *A. calcoaceticus*; ✱, gen. sp. "Close to 13TU"; ✱, gen. sp. "Between 1 and 3". (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

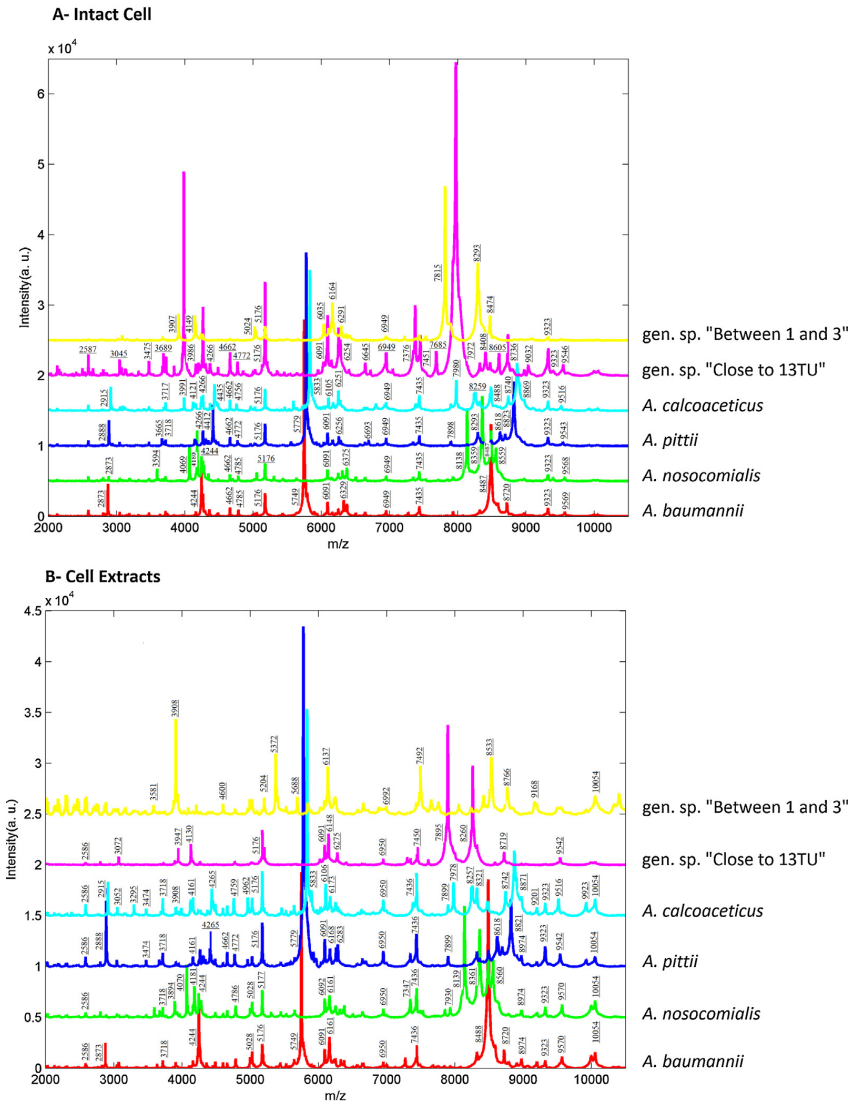

Fig. 2. Peak profiles of the six species in the region of 3000 to 9000 *m*/*z* (obtained from the mean spectra of each species) for IC (A) and CE (B). Legend: , *A. baumannii*; , *A. nosocomialis*; , *A. pittii*; , *A. calcoaceticus*; , gen. sp. "Close to 13TU"; , gen. sp. "Between 1 and 3". (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

In fact, the erroneous identifications of *A. nosocomialis* obtained by Espinal and co-workers were attributed to its absence in the database at that time (MALDI Biotyper Version 2.0SR1 Build 223 database). However, despite the introduction of a new species and the increasing number of bacterial mass profiles of each species increasing the number of correct identifications compared with previous works, one still obtained misidentifications for *A. nosocomialis* and *A. pittii*. Recently, Šedo and co-workers (Šedo et al., 2013) in an attempt to solve the database misidentifications developed an alternative protocol based on the change in matrix composition to improve the rate of correct identifications of the Acb complex species (most of the strains used by Šedo and co-workers were included in this work). Nevertheless, despite the improvements achieved, some issues as the impossibility of automatic mass spectra acquisition or the need of a new reference database, makes this alternative protocol impractical for routine laboratory use. Moreover, strains belonging to species not included in the MALDI Biotyper database will be incorrectly identified with this approach (direct database comparison). As different *Acinetobacter* species may present distinct antimicrobial susceptibility and/or virulence profiles (Grosso et al., 2011; Lai et al., 2012) their correct identification in the clinical context is important. Considering that even with database enlargements or alternative protocols misidentifications still occur and that species not included in the reference database will be falsely allocated to species included in the database, protein profiles database enlargement seems to be not enough, as

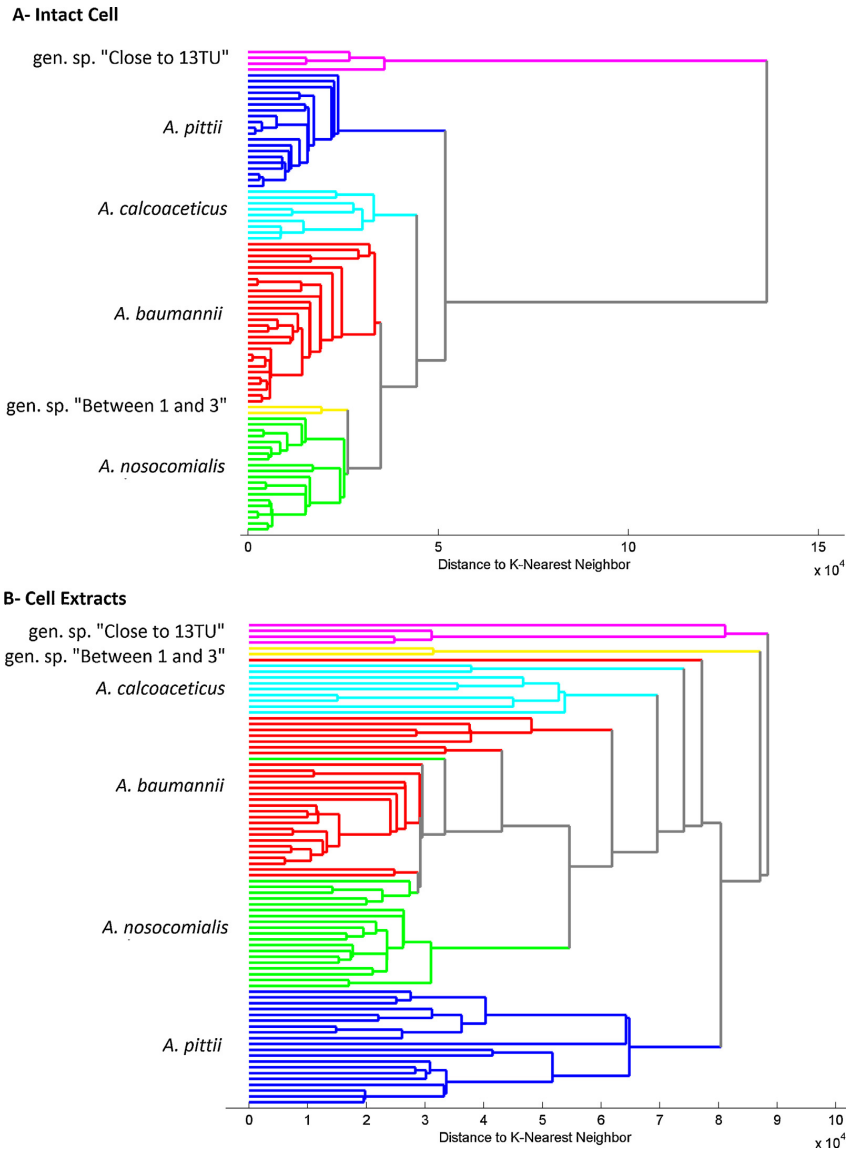

Fig. 3. Dendrogram obtained from the PLSDA model scores with Mahalanobis distance and the agglomerative algorithm distance to k-nearest neighbor for IC (A) and CE (B). Legend: , *A. baumannii*; , *A. nosocomialis*; , *A. pittii*; , *A. calcoaceticus*; , gen. sp. "Close to 13TU"; , gen. sp. "Between 1 and 3". (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

also suggested by others (Espinal et al., 2011), to solve these drawbacks.

A detailed spectra analysis seems to allow the identification of particular genus and species specific *m*/*z* ratios (Fig. 2A and B), some of them already reported by others (Böhme et al., 2010; Espinal et al., 2011; Šedo et al., 2013). However, these *m*/*z* ratios seem to be not enough for unambiguous species identification owing to the divergences of the genus and species specific peaks found in the different works (Böhme et al., 2010; Espinal et al., 2011). Böhme and co-workers pointed three peaks (*m*/*z* 2873, *m*/*z* 5744, *m*/*z* 7429) as being *A. baumannii* specific, however *A. baumannii* was the only species belonging to the *Acinetobacter* genus included in their work. Espinal and co-workers identified *m*/*z* 2873 and *m*/*z* 5750 (*m*/*z* shift of 6 from Böhme's work) as *A. baumannii* species specific. In this study, we found the *m*/*z* 5749 peak (as by Šedo's work); however, in contrast to Böhme's and Espinal's work the 2873 *m*/*z* peak was also found in *A. nosocomialis*. Moreover, we found significant shifts for some peaks of *A. baumannii*, *A. nosocomialis* and *A. pittii* from Espinal's work. These results suggest that species assignment based on few *m*/*z* peaks may be associated with high rates of misidentification. The current limitations described concerning Acb complex species identifications emphasize the need of an

alternative methodology. As demonstrated in this work, the use of specific chemometric methods optimized for each genus could be an alternative for species identification with very similar protein signatures as those belong to the *Acinetobacter* genus. The PLSDA model optimized (Fig. 3A and B) considering IC presents six perfectly individualized clusters corresponding to the six species in agreement with the *rpoB* based identification. With CE species discrimination was poorer maybe due to the fact that fewer proteins were reached during the extracts preparation. In fact, fewer peaks were found for CE (Fig. 2A and B). These results suggests that more complete protein profiles as those obtained with IC could lead to better identifications rates for closely related species. Moreover, using IC sample preparation time and analysis cost can be dramatically reduced.

Considering sensitivity and specificity values (Table 4) obtained for species identification through MALDI-TOF Biotyper database and through the application of an optimized chemometric model to the mass spectra, it is clear that the chemometric approach present higher values for both parameters for both IC and CE. Moreover, with the approach proposed in this work it was also possible to clearly discriminate gen. sp. "Close to 13 TU" and gen. sp. "Between 1 and 3" as distinct species from *A. baumannii*, *A. nosocomialis*, *A. pittii*, and *A. calcoaceticus* which was not possible through the direct MALDI-TOF Biotyper database identification.

Conclusions

In this work we demonstrated that Acb complex species identification by MALDI-TOF MS can be successfully attained by mass spectra fingerprinting approach combined with optimized chemometric methods. A high rate of misidentifications among the Acb complex species were found through direct MALDI Biotyper database identification. However, the PLSDA of mass spectra lead to 100% of correct species identifications using intact cells when compared with *rpoB* species identification. The application of fast, reliable and low cost methods, such as MALDI-TOF MS combined with appropriate chemometric tools would significantly impact bacterial identification in the clinical context.

Conflict of interest statement

The authors have no competing interests.

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Development of FTIR-ATR based model for typing clinically-relevant *Acinetobacter baumannii* clones belonging to ST98, ST103, ST208 and ST218

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Development of a FTIR-ATR based model for typing clinically relevant *Acinetobacter baumannii* clones belonging to ST98, ST103, ST208 and ST218

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ABSTRACT

In this work we developed and validated a FTIR-ATR (Fourier transform infrared spectroscopy with attenuated total reflectance) based model for typing *Acinetobacter baumannii* clinical isolates belonging to ST98, ST208 and ST218 included into the worldwide spread clonal complex (CC) 92 and ST103. FTIR-ATR spectra of seventy-seven previously characterized isolates (Multi Locus Sequence Type-MLST, Pulsed-Field Gel Electrophoresis-PFGE and carbapenem-hydrolyzing class D β-lactamase-CHDL) were acquired and modeled by partial least squares discriminant analysis (PLSDA). The model was tested and successfully validated with a diverse collection of isolates ($n = 148$) recovered from different countries and periods of time belonging to modeled and non-modeled STs.

1. Introduction

Acinetobacter baumannii has emerged in the last decade as an increasingly important nosocomial pathogen, particularly due to its ability to acquire antimicrobial resistance determinants. Outbreaks caused by multidrug resistant *A. baumannii* have been identified worldwide, with several reports of isolates resistant to all available antimicrobial agents [1]. This emergence has been associated with highly successful strains, in particular those belonging to the European Clone II, and more recently ascribed to the clonal complex 92 (CC92), according with the Multi Locus Sequence Typing (MLST) scheme hosted by the Oxford Database [2–5]. Several typing methods have been applied aiming *A. baumannii* population structure characterization, such as Pulsed-Field Gel Electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) analysis, other PCR fingerprinting methods, multiple-locus variable tandem repeat number analysis (MVLA) and MLST schemes. However, the need for immediate interventions in the epidemiological context of *A. baumannii* infection demands for less expensive, less laborious and quicker approaches. Fourier transform infrared spectroscopy (FTIR) coupled with multivariate data analysis (chemometric tools) is considered as a good candidate for this purpose. In a previous work, we [6] demonstrated the ability of FTIR spectroscopy to discriminate among three *A. baumannii* clones (ST98, ST103 and ST208) disseminated in Portuguese hospitals. In this work, we aimed to develop a robust mathematical model for typing *A. baumannii* clones based on Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) and partial least squares discriminant analysis (PLSDA) that could be routinely used. The mathematical model was developed with a collection of 77 *A. baumannii* clinical isolates belonging to four different clones (ST98, ST103, ST208 and ST218) and validated by subjecting it to a test collection of 148 isolates (some of them belonging to the aforementioned STs). These isolates were typed by MLST according with Oxford Database MLST scheme [2] and the STs were compared with the FTIR-ATR model predictions.

2. Material and methods

2.1. Bacterial strains

2.1.1. Modeled isolates (used to develop the model)

Seventy-seven *A. baumannii* clinical isolates were selected among a previously published collection of 318 CHDL-producing

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Acinetobacter spp. recovered from 6 geographically distant Portuguese hospitals (2001–2009). The selected isolates are representatives of the main lineages disseminated in Portugal before 2009: ST98 ($n = 25$); ST103 ($n = 24$); ST208 ($n = 25$; corresponding to a reassignment of ST92 isolates) and ST218 ($n = 3$) [4]. Only three ST218 isolates were found during this period. The main characteristics of the isolates used in this study, including the relationship between carbapenem-hydrolyzing class D β-lactamase (CHDL) content, PFGE types, sequence groups and the STs obtained according with Oxford Database MLST scheme (http://pubmlst.org/abaumannii/) were presented in Table 1.

2.1.2. Test isolates (used to test and validate the model)

One hundred and forty-eight *A. baumannii* clinical isolates from seven different countries and periods of time (1991–2012): France ($n = 6$), Germany ($n = 7$), Czech Republic ($n = 9$), Italy ($n = 11$), Brazil ($n = 12$), Croatia ($n = 9$) and Portugal ($n = 94$) were used to assess the FTIR-ATR based method's robustness. Test isolates included STs used to develop the model and distinct ones (21 different STs) to assess the ability of the model to predict non-modeled STs.

2.2. FTIR-ATR spectral acquisition

FTIR-ATR spectra of all isolates were acquired from an overnight subculture on Mueller Hinton agar exactly after 18 h of grow at 37 °C to avoid spectral interferences due to the age of colony. Different batches of media were used during the experimental work and no spectral differences were observed (data not shown). FTIR-ATR spectra were acquired using a PerkinElmer Spectrum BX FTIR System spectrophotometer equipped with a DTGS detector and a PIKE Technologies Gladi ATR accessory from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1} and 32 scan co-additions. Colonies were directly transferred from the agar plates to the ATR crystal, followed by drying on the optical surface until a thin film was obtained. For each strain, 9 spectra were acquired corresponding to three biological replicates (obtained in three independent days with primary fresh subcultures) and three instrumental replicates (obtained from the same agar plate). Between each isolate measurement a background was acquired.

2.3. Spectra pre-processing and modeling

FTIR-ATR spectra were processed with standard normal variate (SNV) [7] followed by the application of a Savitzky-Golay filter (9 smoothing points, 2nd order polynomial and 2nd derivative) [8] and mean-centring. After pre-processing, spectra were modeled by PLSDA- partial least squares discriminant analysis [9]. In this method, to each isolate spectrum (x_i) was assigned a vector of zeros with the value one at the position corresponding to its ST (y_i) [10]. Isolates were divided in calibration (70%) and test sets (30%). The leave-one-sample-out cross validation strategy (applied on the calibration set only) was adopted to optimize the number of latent variables, the threshold for the probability of class assignment and the confidence limit of the Q statistic (squared residuals) [8]. For each class (ST), a PLSDA prediction threshold level was optimized in order to maximize specificity and sensitivity (a number between 0 and 1). Probabilities of class assignment are calculated based on these thresholds and predictions. More information regarding the structure of the PLSDA model and its application in a similar context was described elsewhere [6].

The cross-validation results showed that a minimum 95% probability was necessary to correctly assign samples to the correct class. Therefore, if the probability for a given ST was higher than 95% then the assignment was considered valid for that ST [11]. If the predicted probabilities for all STs were lower than 95%, the result was said to be non-conclusive and that sample was considered not to belong to any of the four modeled STs (meaning that the sample belong to a ST not included in the model). Besides model estimations, each sample was analyzed accordingly to the model adjustment. This fit was estimated by analyzing the model residuals (statistic Q or sum of squared errors) and comparing these residuals with the confidence limit $Q_{95\%}$ obtained for the calibration set isolates (estimated by cross-validation) [7]. A suitable measure was obtained by dividing that statistic Q by the corresponding 95% confidence limit ($Q/Q_{95\%}$). Values substantially superior to 1 mean that predictions cannot be considered valid once they present significant deviations when compared to any of the modeled STs (that sample is either an outlier meaning that belongs to an ST not considered in this model).

Additionally, projections of test isolates onto the developed discriminant model allowed the identification of these isolates according to one of the four STs or as a distinct ST. A classification can only be considered valid if that isolate spectrum fits the PLSDA model ($Q/Q_{95\%} < 1$) and the probability of class assignment for one class is superior to 95%.

All data analysis was performed in Matlab version 7.9 (MathWorks, Natick, MA) and the PLS Toolbox version 5.5.1 for Matlab (Eigenvector Research, Manson, WA).

2.4. Multi Locus Sequence Type

Multi Locus Sequence Type (MLST) was performed in the test isolates to evaluate the accuracy of the developed mathematical model proposed for typing. MLST was performed according with the conditions proposed by the *A. baumannii* MLST website

Table 1
Portuguese *A. baumannii* isolates used to develop the PLSDA model.

Sequence Type[a] (n)[b]	Sequence group (SG)	ApaI-PFGE type	Acquired CHDL[c]	Hospital[d] (n)	Years	Refs.
98(25)	SG1	A	OXA-40	HGSA (23) CHCB (2)	2001–2008	[4,6]
103(24)	SG4	B, C	OXA-58	HGSA (24)	2001–2004	[4,6]
208(25)	SG1	A4	OXA-23	HA (3) HGSA (13) HPH (2) HST (7)	2006–2009	[4,6]
218(3)	SG1	A5	OXA-23	HGSA (2) VC (1)	2009	This study

[a] Sequence Type (ST) determined according with Oxford Database MLST scheme (http://pubmlst.org/abaumannii/).
[b] *n*, total of isolates.
[c] CHDL, carbapenem-hydrolyzing class D β-lactamase.
[d] HA, Hospital de Amarante (North), HGSA, Hospital Geral de Santo António (North), HPH, Hospital de Pedro Hispano (North), HST, Hospital de São Teotónio (Centre), CHCB, Centro Hospitalar da Cova da Beira (Centre), VC, Centro Hospitalar Póvoa de Varzim – Vila do Conde (North).

(http://pubmlst.org/abaumannii/) sited at the University of Oxford [2].

2.5. Pulsed Field Gel Electrophoresis

Pulsed-Field Gel Electrophoresis (PFGE) was performed in some test isolates whenever there was a need for further clarification on the similarity of isolates. Genome macrorestriction was performed with the ApaI enzyme and pattern analysis was conducted with InfoQuestTM FP v5.4 (BioRad Laboratories, Hercules, CA, USA) using the Dice coefficient with the unweighted pair group method to determine band similarity. Isolates clustering together with >85% similarity levels were considered to belong to the same PFGE type [12–14].

3. Results and discussion

3.1. Spectral overview

FTIR-ATR spectra exhibit the typical shape of intact bacterial spectra containing the absorption bands of lipids (3000–2800 cm^{-1}), proteins/amides I and II (1700–1500 cm^{-1}), mixed

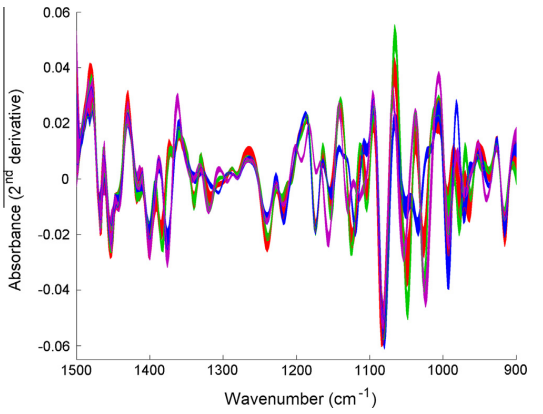


Fig. 1. *Acinetobacter baumannii* FTIR-ATR spectra processed with SNV and Savitzky-Golay (9 points filter size, 2nd degree polynomial, 2nd derivative) corresponding to the mean ± one standard deviations in the region 1500–900 cm^{-1}. Legend: ST98, ST103, ST208 and ST218.

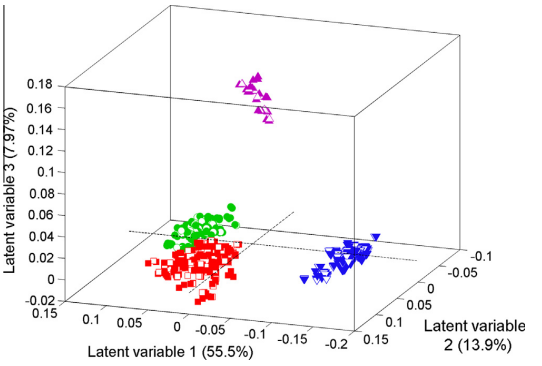


Fig. 2. Score plot corresponding to the first three LVs of the PLSDA regression model using the 1500–900 cm^{-1} spectral region. Legend: ST98 (■); ST103 (●); ST208 (▼); ST218 (▲). Filled and unfilled symbols correspond to calibration and test samples, respectively.

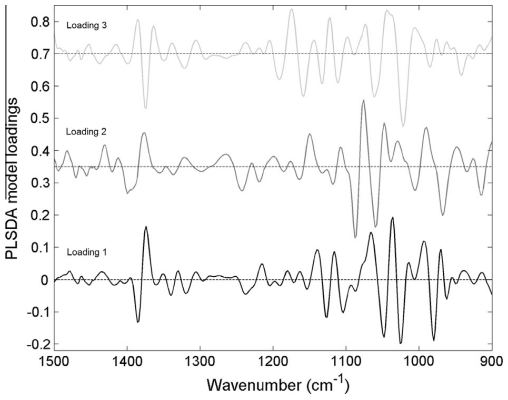


Fig. 3. Loadings of the PLSDA model calibrated with 77 *Acinetobacter baumannii* isolates. Legend: loading 1 (black line), loading 2 (dark gray line) and loading 3 (gray line).

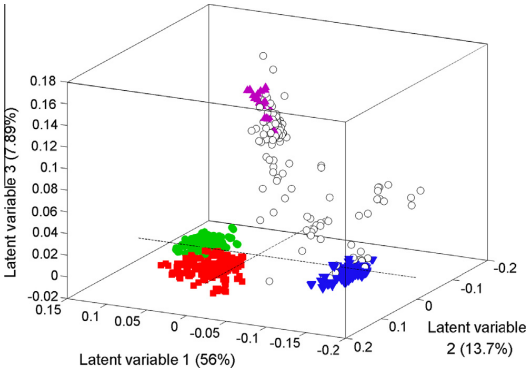


Fig. 4. Score plot corresponding to the three first LVs of the PLSDA regression model with the projected test isolates using the 1500–900 cm^{-1} spectral region. Legend: ST98 (■); ST103 (●); ST208 (▼); ST218 (▲) and test isolates (○).

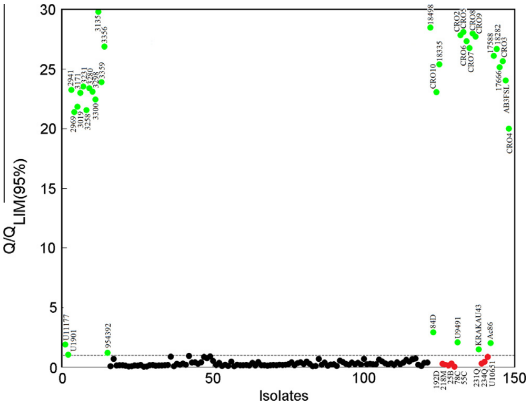


Fig. 5. Normalized squared residuals statistic ($Q/Q_{LIM95\%}$) obtained for the projection of 148 test isolates with indication of the 95% confidence limit. Legend: (●) well predicted isolates as not belonging to the model, (●) badly predicted isolates, (●) well predicted isolates as belonging to the model.

Table 2
Comparison between predicted STs by the FTIR-ATR model and those obtained by MLST scheme. Results are expressed as probabilities of belonging to each ST.

Isolate	Country	ST assignments					
		MLST[a]	FTIR-ATR probabilities				FTIR-ATR predicted ST
			ST98	ST103	ST208	ST218	
U11177	G	15	0.00	0.00	10.34	89.66	N/A
U1901	G	16	0.00	0.00	12.30	87.70	N/A
2941	B	113	0.00	0.00	66.38	33.62	N/A
2969	B	113	0.00	0.00	69.51	30.49	N/A
3019	B	113	0.00	0.00	60.73	39.27	N/A
3171	B	113	0.00	0.00	66.73	33.27	N/A
3231	B	113	0.00	0.00	65.27	34.73	N/A
3258	B	113	0.00	0.00	78.73	21.27	N/A
3280	B	113	0.00	0.00	64.76	35.24	N/A
3298	B	113	0.00	0.00	66.13	33.87	N/A
3300	B	113	0.00	0.00	65.23	34.77	N/A
3135	B	132	0.00	0.00	49.36	50.64	N/A
3359	B	133	0.00	0.00	65.49	34.51	N/A
3356	B	134	0.00	18.87	78.41	2.72	N/A
954392	G	195	0.00	0.00	44.14	55.86	N/A
RC1	CR	208	0.00	0.00	>99.9	0.00	208
RC2	CR	208	0.00	0.00	95.45	4.55	208
RC3	CR	208	0.00	0.00	>99.9	0.00	208
RC4	CR	208	0.00	0.00	>99.9	0.00	208
RC5	CR	208	0.00	0.00	95.90	4.10	208
RC6	CR	208	0.00	0.00	97.72	2.28	208
RC7	CR	208	0.00	0.00	>99.9	0.00	208
RC8	CR	208	0.00	0.00	>99.9	0.00	208
RC9	CR	208	0.00	0.00	>99.9	0.00	208
Ac1	P	208	0.00	0.00	>99.9	0.00	208
Ac2	P	208	0.00	0.00	>99.9	0.00	208
Ac5	P	208	0.00	0.00	>99.9	0.00	208
Ac7	P	208	0.00	0.00	>99.9	0.00	208
Ac20	P	208	0.00	0.00	99.01	0.99	208
Ac21	P	208	0.00	0.00	>99.9	0.00	208
Ac30	P	208	0.00	0.00	>99.9	0.00	208
Ac39	P	208	0.00	0.00	>99.9	0.00	208
Ac68	P	208	0.00	0.00	>99.9	0.00	208
Ac69	P	208	0.00	0.00	>99.9	0.00	208
Ac73	P	208	0.00	0.00	>99.9	0.00	208
Ac78	P	208	0.00	0.00	>99.9	0.00	208
Ac81	P	208	0.00	0.00	>99.9	0.00	208
Ac85	P	208	0.00	0.00	96.28	3.72	208
AB13A	I	218	0.00	0.00	0.00	>99.9	218
1510706	G	218	0.00	0.00	0.00	>99.9	218
147N	I	218	0.00	0.00	0.00	>99.9	218
211P	I	218	0.00	0.00	0.00	>99.9	218
Ac3	P	218	0.00	0.00	0.00	>99.9	218
Ac4	P	218	0.00	0.00	0.00	>99.9	218
Ac6	P	218	0.00	0.00	0.00	>99.9	218
Ac8	P	218	0.00	0.00	0.00	>99.9	218
Ac9	P	218	0.00	0.00	0.00	>99.9	218
Ac10	P	218	0.00	0.00	0.00	>99.9	218
Ac11	P	218	0.00	0.00	0.00	>99.9	218
Ac12	P	218	0.00	0.00	0.00	>99.9	218
Ac13	P	218	0.00	0.00	0.00	>99.9	218
Ac14	P	218	0.00	0.00	0.00	>99.9	218
Ac15	P	218	0.00	0.00	0.00	>99.9	218
Ac16	P	218	0.00	0.00	0.00	>99.9	218
Ac17	P	218	0.00	0.00	0.00	>99.9	218
Ac18	P	218	0.00	0.00	0.00	>99.9	218
Ac19	P	218	0.00	0.00	0.00	>99.9	218
Ac22	P	218	0.00	0.00	0.00	>99.9	218
Ac23	P	218	0.00	0.00	0.00	>99.9	218
Ac24	P	218	0.00	0.00	0.00	>99.9	218
Ac25	P	218	0.00	0.00	0.00	>99.9	218
Ac26	P	218	0.00	0.00	0.00	>99.9	218
Ac27	P	218	0.00	0.00	0.00	>99.9	218
Ac28	P	218	0.00	0.00	0.00	>99.9	218
Ac29	P	218	0.00	0.00	0.00	>99.9	218
Ac31	P	218	0.00	0.00	0.00	>99.9	218
Ac32	P	218	0.00	0.00	0.00	>99.9	218
Ac33	P	218	0.00	0.00	0.00	>99.9	218
Ac34	P	218	0.00	0.00	0.00	>99.9	218
Ac35	P	218	0.00	0.00	0.00	>99.9	218
Ac36	P	218	0.00	0.00	0.00	>99.9	218

(*continued on next page*)

Table 2 (*continued*)

Isolate	Country	ST assignments					
		MLST[a]	FTIR-ATR probabilities				FTIR-ATR predicted ST
			ST98	ST103	ST208	ST218	
Ac37	P	218	0.00	0.00	0.00	>99.9	218
Ac38	P	218	0.00	0.00	0.00	>99.9	218
Ac40	P	218	0.00	0.00	0.00	>99.9	218
Ac41	P	218	0.00	0.00	0.00	>99.9	218
Ac42	P	218	0.00	0.00	0.00	>99.9	218
Ac43	P	218	0.00	0.00	0.00	>99.9	218
Ac44	P	218	0.00	0.00	0.00	>99.9	218
Ac45	P	218	0.00	0.00	0.00	>99.9	218
Ac46	P	218	0.00	0.00	0.00	>99.9	218
Ac47	P	218	0.00	0.00	0.00	>99.9	218
Ac48	P	218	0.00	0.00	0.00	>99.9	218
Ac49	P	218	0.00	0.00	0.00	>99.9	218
Ac50	P	218	0.00	0.00	0.00	>99.9	218
Ac51	P	218	0.00	0.00	0.00	>99.9	218
Ac52	P	218	0.00	0.00	0.00	>99.9	218
Ac53	P	218	0.00	0.00	0.00	>99.9	218
Ac54	P	218	0.00	0.00	0.00	>99.9	218
Ac55	P	218	0.00	0.00	0.00	>99.9	218
Ac56	P	218	0.00	0.00	0.00	>99.9	218
Ac57	P	218	0.00	0.00	0.00	>99.9	218
Ac58	P	218	0.00	0.00	0.00	>99.9	218
Ac59	P	218	0.00	0.00	0.00	>99.9	218
Ac60	P	218	0.00	0.00	0.00	>99.9	218
Ac61	P	218	0.00	0.00	0.00	>99.9	218
Ac62	P	218	0.00	0.00	0.00	>99.9	218
Ac63	P	218	0.00	0.00	0.00	>99.9	218
Ac64	P	218	0.00	0.00	0.00	>99.9	218
Ac65	P	218	0.00	0.00	0.00	>99.9	218
Ac66	P	218	0.00	0.00	0.00	>99.9	218
Ac67	P	218	0.00	0.00	0.00	>99.9	218
Ac70	P	218	0.00	3.61	0.00	96.39	218
Ac71	P	218	0.00	0.00	0.00	>99.9	218
Ac72	P	218	0.00	0.00	0.00	>99.9	218
Ac74	P	218	0.00	0.00	0.00	>99.9	218
Ac75	P	218	0.00	0.00	0.00	>99.9	218
Ac76	P	218	0.00	0.00	0.00	>99.9	218
Ac77	P	218	0.00	0.00	0.00	>99.9	218
Ac79	P	218	0.00	0.00	0.00	>99.9	218
Ac80	P	218	0.00	0.00	0.00	>99.9	218
Ac82	P	218	0.00	0.00	0.00	>99.9	218
Ac83	P	218	0.00	0.00	0.00	>99.9	218
Ac84	P	218	0.00	0.00	0.00	>99.9	218
Ac87	P	218	0.00	0.00	0.00	>99.9	218
Ac88	P	218	0.00	0.00	0.00	>99.9	218
Ac89	P	218	0.00	0.00	0.86	99.14	218
Ac90	P	218	0.00	0.00	0.00	>99.9	218
Ac91	P	218	0.00	0.00	0.00	>99.9	218
Ac92	P	218	0.00	0.00	0.00	>99.9	218
Ac93	P	218	0.00	0.00	0.00	>99.9	218
Ac94	P	218	0.00	0.00	0.00	>99.9	218
18498	F	231	0.00	0.00	67.12	32.88	N/A
84D	I	231	0.00	0.00	66.05	33.95	N/A
CRO10	C	348	0.00	0.00	66.55	33.45	N/A
18335	F	391	0.00	0.00	59.62	40.38	N/A
192D	I	437	10.18	0.00	89.82	0.00	N/A
218M	I	437	0.00	0.00	>99.9	0.00	208
25B	I	437	0.00	0.00	>99.9	0.00	208
55C	I	437	0.00	0.00	>99.9	0.00	208
78C	I	437	0.00	0.00	>99.9	0.00	208
U9491	G	439	0.00	0.00	81.92	18.08	N/A
CRO2	C	441	0.00	0.00	60.71	39.29	N/A
CRO5	C	441	0.00	0.00	64.64	35.36	N/A
CRO6	C	441	0.00	0.00	63.06	36.94	N/A
CRO7	C	441	0.00	6.29	60.80	32.91	N/A
CRO8	C	441	0.00	0.00	61.96	38.04	N/A
CRO9	C	441	0.00	0.00	61.32	38.68	N/A
KRAKAU43	G	441	70.63	0.00	29.37	0.00	N/A
231Q	I	513	0.00	0.00	0.00	>99.9	218
234Q	I	513	0.00	0.00	0.00	>99.9	218
U10651	G	514	50.23	0.00	0.00	49.77	N/A
Ac86	P	515	0.00	0.00	11.47	88.53	N/A
17588	F	732	0.00	5.87	29.64	64.49	N/A

Table 2 (*continued*)

Isolate	Country	ST assignments					
		MLST[a]	FTIR-ATR probabilities				FTIR-ATR predicted ST
			ST98	ST103	ST208	ST218	
18282	F	732	0.00	9.46	27.96	62.58	N/A
17666	F	733	0.00	0.00	34.77	65.23	N/A
CRO3	C	740	0.00	27.02	57.94	15.04	N/A
AB3FSL	I	New[b]	0.00	0.00	54.85	45.15	N/A
CRO4	C	New[b]	0.00	0.00	58.97	41.03	N/A

[a] Sequence type obtained in this study by Bartual MLST scheme (http://pubmlst.org/abaumannii/).
[b] Alelic profiles: AB3FSL (2-21-107-32-26-198-5); CRO4 (10-12-4-11-4-102-5); N/A- not assigned to any of the four modeled STs; CR-Czech Republic, P-Portugal, G-Germany, I-Italy, F-France, B-Brazil and C-Croatia.

region of phospholipids/DNA/RNA (1500–1185 cm^{-1}), polysaccharides (1185–900 cm^{-1}) and the fingerprint region (900–600 cm^{-1}) [15,16]. The phospholipids/DNA/RNA and carbohydrates regions (Fig. 1) exhibiting the highest spectral variance, have already been used with great success for the discrimination of *A. baumannii* clones by FTIR [6] and were chosen for all subsequent analysis.

3.2. Model development

A collection of 77 Portuguese isolates belonging to four different STs (ST98, ST103, ST208 and ST218) were used to develop the PLSDA model based on FTIR-ATR spectroscopy. This dataset was divided in two subsets: one for calibration and one for testing. This division ensured that all sample replicates were kept together and that a proportional number of representative STs existed in both sets. The best number of latent variables (LVs) was estimated from the calibration set using the leave-one-out cross validation strategy as described in a previous work [6]. Results indicated that the best model included three LVs. To verify the predictive ability of the model, the test set spectra were projected onto the model. It is possible to verify on the model scores plot (Fig. 2) a very good ability to discriminate between the four STs (ST98, ST103, ST208 and ST218). ST208 was discriminated from ST98, ST103 and ST218 by the first latent variable of the PLSDA model (corresponding to model loading 1, Fig. 3), ST98 was discriminated from ST103 by the second latent variable (corresponding to the second loading) and ST218 from ST98 and ST103 by the third latent variable (corresponding to model loading three). It is of note that PLSDA model loadings are somehow similar to those obtained in our previous work [6] being the more pronounced differences between the four clones observed in the regions 1400–1350 cm^{-1} and 1200–950 cm^{-1}. The herein PLSDA developed model presents a higher discrimination ability when compared with our previous work due to a higher number of isolates and/or STs and to some minor adjustments in data preprocessing.

Moreover, the projection of the test set isolates shows an excellent agreement with calibration isolates on the scores plot (Fig. 2). Considering the test set it was possible to identify a 99.3% rate of correct predictions for the ST [6].

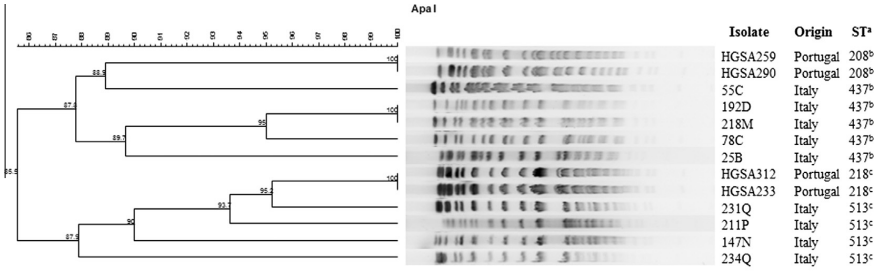
3.3. Model test and validation

Spectra of the test isolates were projected on the previously built PLSDA model for ST prediction (Fig. 4) and the residuals statistic for each projected isolate were represented in Fig. 5. The model predictions, expressed as probabilities of belonging to a certain ST, together with the MLST results are summarized in Table 2.

According to the projections in the PLSDA model, visualized as model scores (Fig. 4), all the isolates were predicted as belonging to ST208, ST218 or to diverse STs not included in the developed model. Moreover, none of the test isolates was assigned as ST98 or ST103 which was in agreement with the respective probabilities (lower than 95% for ST98 and ST103, Table 2) and the MLST results.

All ST208 and ST218 isolates (106/106) were well predicted by FTIR-ATR based model with probabilities higher than 95% and presented a $Q/Q_{95\%}$ lower than 1 supporting the reliability of the model predictions. Among the isolates from diverse STs, thirty-five (35/42) were well predicted as not belonging to any of the modeled STs (with probabilities lower than 95% for all the modeled STs). Moreover, all but one isolate (U10651) presented a $Q/Q_{95\%}$ value superior to 1 meaning that they didn't fit the model and belong to a different ST (Fig. 5).

Nevertheless, five ST437 isolates were predicted as ST208 and two ST513 were predicted as ST218 (Table 2), with probabilities higher than 95% (except 192D for which the probability of belonging to ST208 was 89.8%) and a $Q/Q_{95\%}$ value lower than 1. It is of



[a]ST-sequence type determined according with MLST scheme from A. baumannii MLST website, University of Oxford; [b]ST208 and ST437 are SLV for the gyrB gene; [c]ST218 and ST513 are SLV for the recA gene. PFGE pattern analysis was conducted with InfoQuestTM FP v5.4 (BioRad Laboratories, Hercules, CA, USA). The similarity percentage of profiles was calculated applying the Dice coefficient. The optimization and position tolerance for band analysis were set at 1%.

Fig. 6. Dendrogram of ApaI-PFGE fingerprints analysis for single locus variant isolates from ST208/ST437 and ST218/ST513.

note that ST437/ST208 and ST513/ST218 are single locus variants for the *gyr*B and *rec*A genes, respectively. In order to clarify the isolates similarity of these two pairs of SLV, PFGE experiments were performed (Fig. 6). PFGEs profiles exhibited similarity coefficients >85%, which is considered the cut-off value to belong to the same *Acinetobacter* PFGE clone (13, 14). The PFGE patterns comparison between the SLV ST208/ST437 revealed similarity coefficient of 87.8%, and between the SLV ST218/ST513 the similarity coefficient was 87.9%. Moreover, isolates belonging to the same ST presented PFGE patterns with similarity coefficients values varying between 87.8% and 100% (Fig. 6).

These results clearly validate the FTIR-ATR model to discriminate *A. baumannii* clones belonging to ST98, ST103, ST208 and ST218 resulting in a reproducible and highly discriminatory methodology that could be used in routine *A. baumannii* typing. Competing spectroscopic techniques as Raman spectroscopy seems to exhibit a lower discrimination power concerning bacteria typing at subspecies level presenting less sensitivity and specificity [17,18]. Moreover, to the specific purpose of this work, this technique presents several advantages when compared with whole genome sequencing: rapidity, a significantly lower cost and less data handling.

4. Conclusions

FTIR-ATR spectroscopy detected specific molecular features on clinically relevant *A. baumannii* clonal lineages assigned according to Oxford MLST scheme to ST98, ST103, ST208 and ST218 stressing the significance of these lineages and reinforcing the epidemiological interest in their identification.

This spectroscopic technique coupled with appropriate chemometric methods showed a good discrimination ability of clinically relevant *A. baumannii* clonal lineages providing a rapid, easy and low cost methodology for hospital surveillance of *A. baumannii* at a large-scale basis, which could improve infection control and individual patient decisions in a clinical context.

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Unsuitability of MALDI-TOF MS to discriminate *Acinetobacter baumannii* clones under routine experimental conditions

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MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) is now in the forefront for routine bacterial species identification methodologies, being its value for clonality assessment controversial. In this work we evaluated the potential of MALDI-TOF MS for assisting infection control by depicting *Acinetobacter baumannii* clones. Mass spectra of 58 *A. baumannii* clinical isolates belonging to the worldwide spread lineages (ST98, ST103, ST208, and ST218) isolated in our country, were obtained and analyzed with several chemometric tools (pseudo gel views, *peakfind* function, and partial least squares discriminant analysis). The clonal lineages were obtained using the "Oxford" scheme, belonging ST98, ST208, and ST218 to the international clone II and ST103 to an epidemic clonal lineage (SG5). Additionally, mass spectra of a highly diverse international collection of 38 isolates belonging to 22 sequence types (STs) were obtained for further comparisons. Pseudo gel views and direct peak pattern analysis did not allow the discrimination of *A. baumannii* isolates belonging to ST98, ST103, ST208, or ST218. Moreover, a partial least square discriminant analysis of the mass spectra considering two spectral ranges (2–20 kDa and 4–10 kDa) revealed a poor degree of discrimination with only 64.6 and 65.8% of correct ST assignments, respectively. Also, mass spectra of the international isolates ($n = 38$, 22STs) revealed a very congruent peak pattern among them as well as among the four lineages included in this work. Despite the increasing interest of MALDI-TOF MS for bacterial typing at different taxonomical levels, we demonstrated, using routine experimental conditions, the unsuitability of this methodology for *A. baumannii* clonal discrimination.

Keywords: *Acinetobacter baumannii*, typing, MALDI-TOF MS, subspecies, chemometrics, sequence type

Introduction

During the last decade, the rate of nosocomial infections caused by multidrug-resistant *Acinetobacter baumannii* (MDRAB) has increased worldwide. In particular, the growing number

of carbapenem-resistant *A. baumannii* isolates, mainly due to the production of carbapenem-hydrolyzing class D β-lactamases (CHDLs) jeopardizes the treatment of infections caused by this agent (Higgins et al., 2010). The quick and reliable clonality assessment is crucial to rapidly trace its dissemination, assist antibiotherapy, and implement measures to constrain its dissemination.

Pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) analysis, multilocus sequence typing (MLST), Diversilab^{TM} typing, other PCR fingerprinting methods, and multiple-locus variable tandem repeat number analysis (MVLA) are among the current methods used for genotyping bacteria (Grosso et al., 2011b; Karah et al., 2012; Zander et al., 2012; Zarrilli et al., 2013). PCR and sequence-based methods which describe isolates numerically are easy to use in international networks. Specifically for *A. baumannii,* Sequence Groups (SGs) identification or Trilocus (Turton et al., 2007) sequence-based typing (3LST – selectively amplifies alleles of *omp*A, *csu*E, and bla_{OXA-51} genes), $bla_{OXA-51-like}$ genotyping and two MLST schemes[1] ("Oxford" and Institute Pasteur) are available. The 3LST, $bla_{OXA-51-like}$ genotyping, and Diversilab^{TM} typing (which consists in a repetitive extragenic palindromic PCR – rep-PCR) allow the rapid identification of the main multidrug-resistant *A. baumannii* lineages, being already established the concordance between some of these methods (Zarrilli et al., 2013). The MLST schemes share three loci but reveal different discriminatory abilities, with our data pointing for a higher resolution, congruent to PFGE analysis and CHDL content, of the "Oxford" scheme (Grosso et al., 2011b). MLST analysis in several collections resulted in the recognition of major clonal complexes (CCs) and sequence types (STs) responsible for antimicrobial resistance dissemination, such as CC92 (ST92, ST208, ST218), CC109 (ST109), CC103 (ST103, ST133), and CC113 (ST113) according with the Oxford scheme (Zarrilli et al., 2013).

However, most of these methods are too expensive and/or time consuming. Spectroscopic techniques might constitute reliable alternatives for bacterial typing at different taxonomic levels, with variable degrees of success reported from their application to several microorganisms (Mencacci et al., 2013; Šedo et al., 2013; Vaz et al., 2013; Branquinho et al., 2014a,b; Novais et al., 2014; Sousa et al., 2014a). Recently, we developed and validated a mathematical model for typing *A. baumannii* clones, most of them included in this study, based on spectra obtained by a competitive spectroscopic technique, Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR), that could be routinely used (Sousa et al., 2014b). Moreover, using matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and chemometrics we were able to circumvent difficulties associated with the discrimination by MALDI-TOF MS of *Acinetobacter* species within the *A. baumannii-calcoaceticus* complex (Böhme et al., 2010; Espinal et al., 2011; Sousa et al., 2014a). In which concerns the suitability of MALDI-TOF MS for bacterial typing at subspecies level, controversial data is available (Barbuddhe et al., 2008; Dieckmann and Malorny, 2011; Gekenidis et al., 2014; Lasch et al., 2014; Novais et al., 2014; Wolters et al., 2011).

In this work, we evaluated the ability of MALDI-TOF MS combined with several chemometric tools for assisting infection control by depicting *A. baumannii* clones.

Materials and Methods

Rational of the Study

To evaluate the potential of MALDI-TOF MS to discriminate *A. baumannii* clones, mass spectra of 58 Portuguese clinical isolates belonging to ST98, ST103, ST208, and ST218 according with the "Oxford" scheme were obtained and analyzed with several chemometric tools. Mass profiles analysis was performed by different approaches: (i) spectral overview on the basis of pseudo gel views; (ii) analysis of the mass peak pattern according the number of peaks found with the Matlab function *peakfind*; and (iii) partial least squares discriminant analysis (PLSDA). The last two approaches also allowed the estimative of correct predictions for each ST and the PLSDA model was performed considering the entire (2–20 kDa) and a selected (4–10 kDa) spectral range where the majority of the mass peaks (about 90%) were located. Additionally, mass spectra of 38 isolates from different countries and belonging to 22 distinct STs were obtained and the respective pseudo gel views compared among each other and with the ones of ST98, ST103, ST208, and ST218 isolates. We further explored a possible correlation between the mass profiles of the isolates tested and clustering/classification obtained with other methods for major lineages *A. baumannii* identification, as AFLP, SG typing, and the MLST scheme of Institute Pasteur.

Bacterial Isolates

Fifty-eight *A. baumannii* clinical isolates were selected among a previously published collection of 318 CHDL-producing *Acinetobacter* spp. recovered from six geographically distant Portuguese hospitals (2001–2012). The selected isolates are representatives of the main lineages disseminated in Portugal before 2012: ST98 ($n = 10$); ST103 ($n = 9$); ST208 ($n = 17$; corresponding to a reassignment of ST92 isolates), and ST218 ($n = 22$; Grosso et al., 2011a,b; Sousa et al., 2014b). Additionally, 38 isolates belonging to 22 different STs were used for further comparisons. The main characteristics of the isolates used in this study, including the relationship between CHDL content, PFGE types, SGs, and the STs obtained according with Institute Pasteur and "Oxford" scheme[1] among others were presented in **Table 1**.

MALDI-TOF MS Experiments

Mass spectra were obtained from cell extracts prepared according the manufacturer instructions. Briefly, overnight cultures in Muller-Hinton agar were suspended in HPLC water and treated with ethanol (75%). After centrifugation and removal of the supernatant, cells were extracted with 25 μL of 70% formic acid followed by addition of 25 μL of acetonitrile and vortexing at 2000 rpm for 1 min. Samples were spotted onto MALDI ground steel target (AnchorChip) followed by drying and the

[1] http://pubmlst.org/abaumannii/

addition of 1 μL of the chemical matrix (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% of acetonitrile and 2.5% of trifluoroacetic acid). Spectra were randomly obtained from blind samples in the linear positive mode at a laser (nitrogen) frequency of 20 Hz in the range of 2–20 kDa with a Microflex III instrument (Bruker Daltonic, Bremen, Germany). Each recorded spectrum is the result of six series of 40 single laser shots in different locations. The experiments were performed in quadruplicate using four distinct spots of the MALDI target (instrumental replicates) at least in two different days (biological replicates) with two different bacterial cultures or extracts. External calibration of the mass spectra was performed using *Escherichia coli* DH5 alpha standard peaks (BTS).

Data Analysis

Due to the large amount of data generated by MALDI experiments, mean spectrum for each isolate was generated from the instrumental and biological replicates and considered for further analysis. Zero-line and low S/N ratio mass spectra were not considered to the average. The pseudo gel views were generated by the dedicated Matlab-based software MicrobeMS[2] that provides direct access to the spectra of Bruker's proprietary file format. In these gel views the intensities are gray-scaled (log scale) being the mass/charge ratios (m/z) the abscissa and spectral indices the ordinate values. In these bar code spectra only the information of peak presence or absense is employed, while the peak intensity is neglected.

Mass spectra were also analyzed with the *peakfind* function of the PLS Toolbox for Matlab (arguments: 9- number of points in Savitzky–Golay filter, 6- tolerance on the estimated residuals; peaks heights are estimated to be >tolerance*residuals) and 19-window width for determining local maxima to evaluate the intra and inter-ST variability among the four distinct STs. The method starts by estimating the peak mass-to-charge ratios of each ST. For this task, spectra of all isolates belonging to one ST were averaged, the result was submitted to the peak identification method and the peak locations were stored in a vector generating a "peak prototype" for each ST. This method was repeated for all STs. Then, the same peak identification method was run for each isolate individually. Peak positions of each "ST prototype"

[2]http://www.mara-ms.com

TABLE 1 | Epidemiological details of the *Acinetobater baumannii* isolates included in this study.

ST (CC)[b]	ST (CC)[c]	SG (AFLP)[a]	*n*[d]	PFGE[e]	CHDL[f]	Country	Years	Reference
98 (92)	2 (2)	SG1 (II)	10	A	OXA-40	Portugal	2001–2008	Grosso et al. (2011b), Sousa et al. (2014b)
208 (92)	2 (2)	SG1 (II)	17	A4	OXA-23	Portugal	2006–2010	Grosso et al. (2011b), Sousa et al. (2014b)
218 (92)	2 (2)	SG1 (II)	22	A5	OXA-23	Portugal	2010	Sousa et al. (2014b); This study
103 (103)	15 (15)	SG5	9	B, C	OXA-58	Portugal	2001–2004	Grosso et al. (2011b), Sousa et al. (2014b)
15		G1ompA/G2csuE	1	O	–	Germany	1991	Seifert et al. (2005), Sousa et al. (2014b)
16		SG2 (I)	1	L	–	Germany	1991	Seifert et al. (2005), Sousa et al. (2014b)
113 (113)	79 (79)	SG1 (II)	4	D	OXA-23	Brazil	2006–2007	Grosso et al. (2011a)
132	162	$G1_{OXA66}/G2_{ompA}/_{csuE}$	2	F	OXA-23	Brazil	2007	Grosso et al. (2011a)
133 (103)	15 (15)	SG4	3	E	OXA-23	Brazil	2006–2007	Grosso et al. (2011a)
134	316	$G2_{ompA}/_{OXA69}$	1	K	OXA-23	Brazil	2007	Grosso et al. (2011a)
195		SG1 (II)	1	P	OXA-23	Germany	2010	Sousa et al. (2014b)
231		$G2_{OXA-69}/_{ompA}$	2	J, X	OXA-23	Germany, Italy	2004–2012	Sousa et al. (2014b)
236		SG1 (II)	1	T	–	Italy	2012	This study
348		SG1 (II)	1	R	OXA-72	Croatia	2001–2007	Goic-Barisic et al. (2011), Sousa et al. (2014b)
391		$G1_{ompA}$	1	U	–	Germany	2012	Sousa et al. (2014b)
437 (92)	2 (2)	SG1 (II)	5	A4	OXA-58	Italy	2004–2008	D'Arezzo et al. (2009), Sousa et al. (2014b)
439		SG2 (I)	1	M	–	Germany	1991	Seifert et al. (2005), Sousa et al. (2014b)
441		SG2 (I)	4	H	–	Germany, Croatia	2001–2007	Sousa et al. (2014b)
513 (92)	2 (2)	SG1 (II)	2	A5	OXA-23	Italy	2007–2009	D'Arezzo et al. (2011), Sousa et al. (2014b)
514		$G1_{csuE}/G2_{OXA-69}$	1	N	–	Germany	1991	Sousa et al. (2014b)
515			1	I	–	Portugal	2010	Sousa et al. (2014b)
732		$G1_{ompA}$	2	V, W	–	Germany	2012	Sousa et al. (2014b)
733		$G1_{ompA}$	1	Y	OXA-23	Germany	2012	Sousa et al. (2014b)
740		SG2 (I)	1	Q	–	Croatia	2001–2007	Sousa et al. (2014b)
775		SG2 (I)	1	G	–	Croatia	2001–2007	Sousa et al. (2014b)
776		$G1_{OXA66}/_{ompA}/G2_{csuE}$	1	S	–	Italy	2012	Sousa et al. (2014b)

[a]*SG, sequence group typing (PCR-based sequence groups based on ompA, csuE, and* bla_{OXA-51} *genes); AFLP, amplified fragment length polymorphism correspondence to international clones I–III);* [b]*Sequence Type (ST) and Clonal Complex (CC) determined according with the "Oxford" scheme;* [c]*Sequence Type (ST) and Clonal Complex (CC) determined according with Institut Pasteur MLST scheme;* [d]*n total of isolates;* [e]*ApaI-Pulsed Field Gel Electrophoresis type;* [f]*CHDL, acquired carbapenem-hydrolyzing class D β-lactamase.*

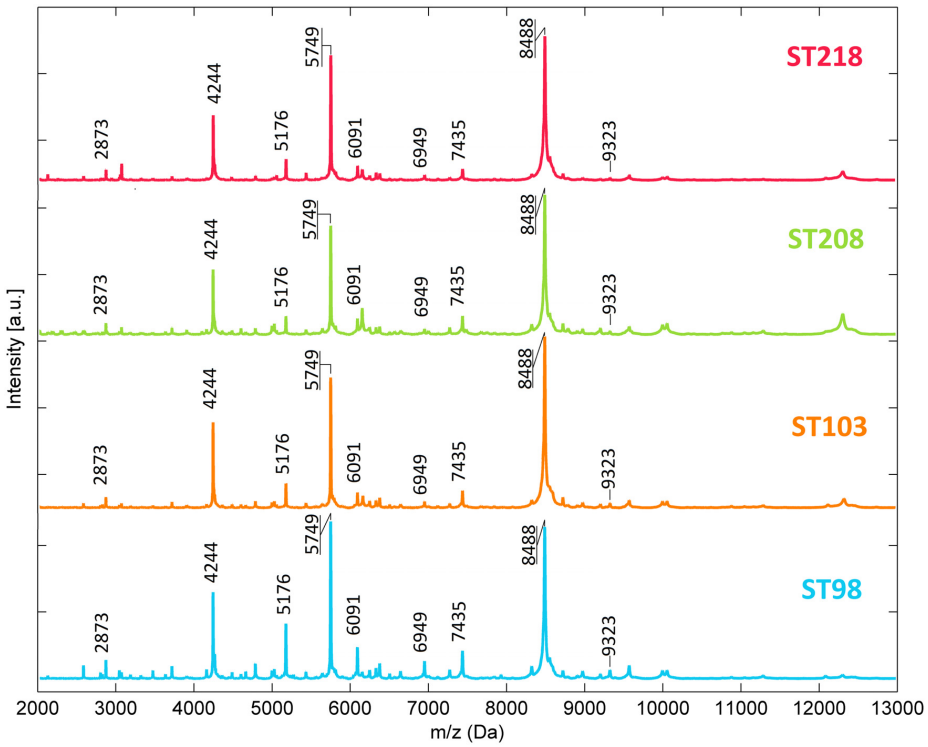

FIGURE 1 | Mass profiles of ST98, ST103, ST208, and ST218 isolates in the region of 2–13 kDa [obtained from the mean spectra of each sequence types (ST)].

were compared with peak positions of each isolate. When a "ST prototype" peak location matched a peak location of an isolate, a value 1 was assigned for that peak; otherwise a value 0 was assigned. This procedure creates a vector of 0s and 1s for each pair "ST prototype"/isolate. Note that peak locations were considered to match if they were located within a mass-to-charge ratio difference lower than 7 m/z units [if for a certain peak location n, $|\ \mathrm{m/z(prototype)_n} - \mathrm{m/z(sample)_n}\ | < 7$, that peak is considered to match]. For each isolate, a percentage of matching peaks was estimated for each ST. Isolates were considered to belong to the ST yielding the highest percentage of peak matches.

For clustering purposes spectra were analyzed by PLSDA after the pre-processing mean-centring (Savitzky and Golay, 1964; Geladi and Kowalsky, 1986; Barker and Rayens, 2003). This pre-processing method allows removing the influence of different sample amounts and/or equipment variations in the peaks intensity. In PLSDA, to each known sample (x_i) is assigned a vector of 0s with the value 1 at the position corresponding to its ST (y_i). The structure of the PLSDA model is described by Eqs 1 and 2. Model loadings (P and Q) and corresponding scores (T and U) are obtained by sequentially extracting the components or latent variables (LVs) from matrices X (the spectra) and Y (the matrix codifying the STs).

$$X = TP^t + E \tag{1}$$

$$Y = UQ^t + F \tag{2}$$

The algorithm correlates the scores of each block (T and U), yielding an internal regression matrix. This internal regression can be transformed on a regression matrix (B). In this case, the regression matrix is composed by three vectors: one regression vector corresponding to each ST. E and F are the residual matrices and depend on the number of LV selected. Predictions for new samples are obtained by multiplying a new spectrum (x_{new}) by the regression matrix (B).

$$y_{new} = x_{new}B \tag{3}$$

The prediction ($y_{new} = [y_{new,1}, y_{new,2}, \ldots, y_{new,n}]$) is then converted in a class assignment. In PLSDA a probability value for each assignment is estimated for each sample. The model

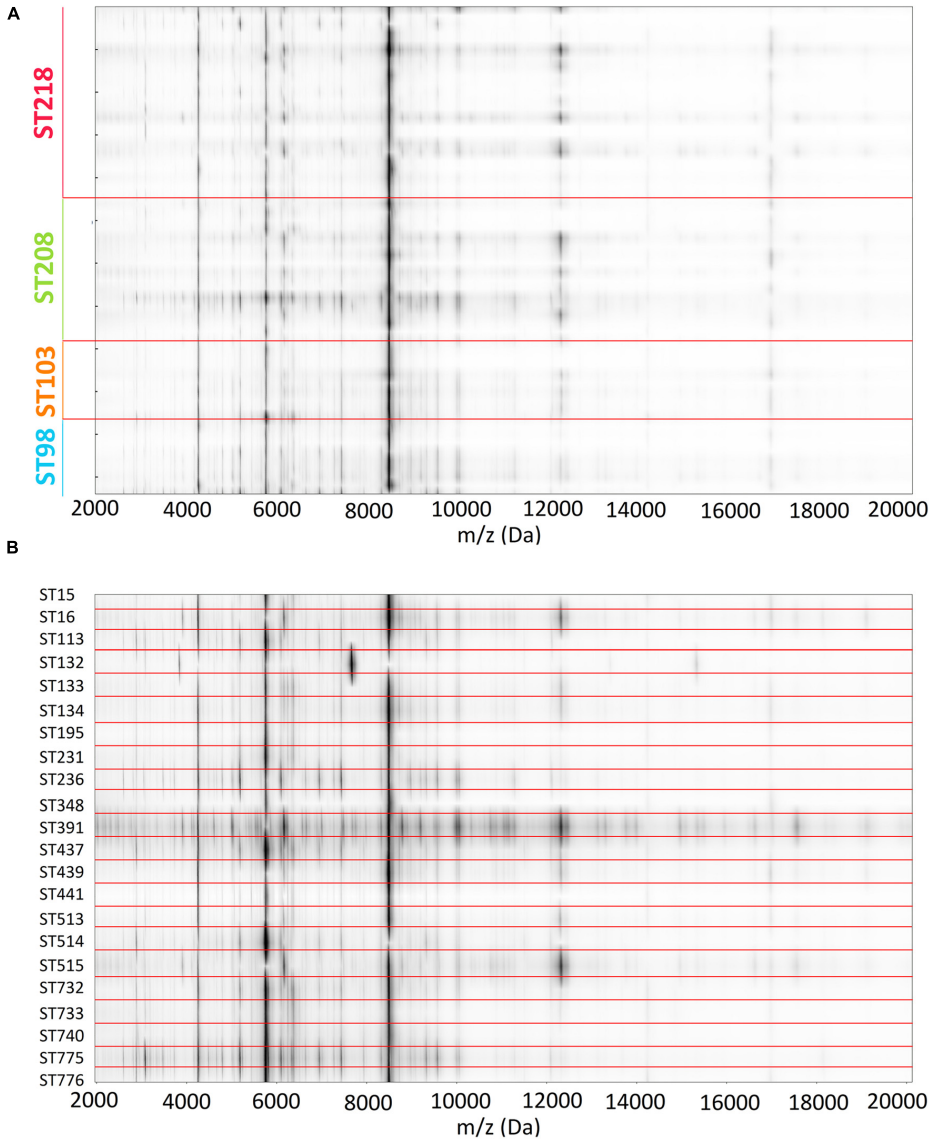

FIGURE 2 | Pseudo gel view representation obtained from the average mass spectra of each *Acinetobacter baumannii* isolate of ST98, ST103, ST208, and ST218 (A) and of *A. baumannii* isolates of the international collection (B).

number of LV was optimized using the leave-one-sample-out cross-validation procedure in order to prevent overfitting. All chemometric models were performed in Matlab version 7.4 Release 2007a (MathWorks, Natick, MA, USA) and PLS Toolbox version 4.2.1 for Matlab (Eigenvector Research, Manson, WA, USA).

Results

Spectral Overview

Mean mass profiles of the isolates belonging to the four studied STs are presented in **Figure 1**. A very similar peak pattern can be found among the isolates of a single ST (data not shown) and

among the four STs with almost no differences between them. The common peaks found among the four STs are summarized in the figure. **Figure 2** exhibits the pseudo gel views generated with MicrobeMS software considering all the isolates included in this study (ST98, S103, ST208, ST218 plus the 22 STs of the international collection). A high degree of consistency in the peak pattern can be found among the isolates of a single ST but also among the four STs (**Figure 2A**). It was impossible to obtain any degree of isolate discrimination according the ST solely based in the presence and/or absence of specific mass peak profiles even considering isolates epidemiologically unrelated and belonging to diverse STs (**Figure 2B**).

Peakfind Function of Matlab

Supplementary Figure S1 summarizes the peak positions founded with the *peakfind* function in the mass spectra from all isolates. No peaks were found above 17 kDa. Similarly to the observations in the pseudo gel view analysis, a very consistent peak pattern was observed among all the isolates with a low inter and intra-ST spectral variability. The comparison of each isolate peak profile with the four mean-ST peak profiles (see Materials and Methods) was used for estimating the ST of each isolate, **Figure 3**. Nevertheless, it was only possible to correctly predict the ST for 58.6% of the isolates. ST103 isolates were always correctly predicted; however, 19/58 isolates were erroneously predicted as ST103, meaning a high sensitivity (9/9 = 100%) and low specificity (30/51 = 58.8%) for the ST103 prediction. Moreover, it was possible to correctly predict 70% of the ST98 isolates; 41.2% of the ST208 isolates and 50% of the ST218 isolates.

Partial Least Squares Discriminant Analysis

The PLSDA models developed considering the entire (2–20 kDa) and a selected (4–10 kDa) spectral range are presented in **Figures 4A,B**, respectively. Considering the entire spectral range (**Figure 4A**) it was not possible to clearly discriminate the four STs as no individualized clusters could be found in the score map of the model. The PLSDA model was able to correctly predict the STs of 64.6% of the isolates (**Table 2**) being ST103 the one with a larger percentage of correct predictions (75.7%). The worst cases were observed for ST98 and ST208 for which plus than 40% of the isolates were erroneously predicted. Similarly, the PLSDA model obtained with the selected spectral range (4–10 kDa) did not allow the discrimination of the four STs (**Figure 4B**) still being ST103 the best predicted (73.7%), **Table 2**. Moreover, the total percentage of correct predictions was slightly higher (65.8%) for each of the three remaining STs considering the 4–10 kDa range.

Partial least squares discriminant analysis models were also developed (whenever possible and according to the available information, please see **Table 1**) to correlate the mass profiles of the isolates with the results obtained from less discriminatory typing methods as AFLP, SG, and MLST scheme of Institute Pasteur. However, the clustering analysis was not congruent with any of these methods (data not shown).

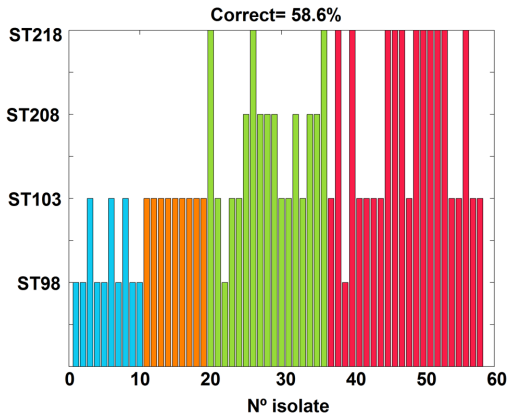

FIGURE 3 | Sequence type assignments for all isolates considering the spectral matching method. Colors identify the real ST of each isolate and bars correspond to the method's ST predictions (58.6% of correct predictions). Legend: ST98, ST103, ST208, and ST218.

Discussion

In the last years an impressive growing number of studies unveiling the MALDI-TOF MS potential for routine bacterial species identification has been published (Böhme et al., 2010; Carbonnelle et al., 2010; Branquinho et al., 2014a,b; Sousa et al., 2014a). However, the suitability of this mass spectrometry technique for bacterial discrimination at the subspecies level has been barely explored, with contradictory outcomes for particular species (Barbuddhe et al., 2008; Dieckmann and Malorny, 2011; Wolters et al., 2011; Gekenidis et al., 2014; Lasch et al., 2014; Novais et al., 2014). The goal of this work was to assess the ability of MALDI-TOF MS to depict *A. baumannii* clones, with particular interest for the worldwide spread lineages (ST98, ST103, ST208, and ST218), contributing to a better understanding of the capabilities and limitations of this technique in bacterial typing. Analysis of the mass spectra of the four STs was attempted using three approaches and revealed some mass-to-charge ratios already identified as *Acinetobacter* genus and *A. baumannii* species-specific (Böhme et al., 2010; Espinal et al., 2011; Šedo et al., 2013; Sousa et al., 2014a). However, the high similarity among mass profiles of the *A. baumannii* lineages analyzed prevented the STs discrimination either by peak pattern direct analysis (**Figure 1**) or based on the presence/absence of specific peaks depicted in the pseudo gel views (**Figure 2A**). Moreover, attempting to assign a ST based on the comparison of each isolate's mass profile with the mean-ST profile also resulted in a low percentage of correct identifications (58%, **Figure 3B**). Previous studies, including from our group (Novais et al., 2014; Sousa et al., 2014a), have demonstrate that the use of specific and optimized chemometric tools in MALDI-TOF MS data analysis improves the bacterial discrimination derived from this spectroscopic methodology. In this context, we attempted to discriminate the isolates

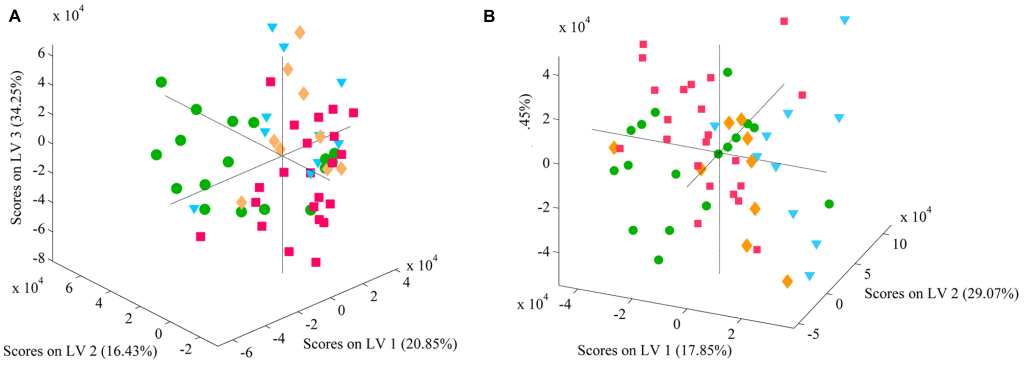

FIGURE 4 | Score plot corresponding to the first three latent variables (LVs) of the partial least squares discriminant analysis (PLSDA) regression model using the entire, 2–20 kDa, (A) and a selected, 4–10 kDa, (B) spectral range. Legend: ▼ ST98, ♦ ST103, ● ST208, and ■ ST218.

TABLE 2 | Confusion matrix for both of the *A. baumannii* PLSDA discrimination models, considering 20 LV (values are in %).

		ST obtained with MLST[a]							
		Spectral range 2–20 kDa				Spectral range 4–10 kDa			
		ST98	ST103	ST208	ST218	ST98	ST103	ST208	ST218
ST predicted by MALDI-TOF MS	ST98	56.2	5.7	12.6	14.4	56.6	8.1	13.1	12.7
	ST103	3.5	75.7	5.3	2.3	2.8	73.7	4.7	3.2
	ST208	17.2	8.8	56.8	13.7	19.5	10.0	60.6	13.6
	ST218	23.1	9.8	25.3	69.6	21.1	8.2	21.5	70.4

[a]*Sequence type determined according with "Oxford" scheme (http://pubmlst.org/abaumannii/).*

with a PLSDA analysis considering two distinct mass ranges (**Figures 4A,B**). Although the degree of discrimination slightly improved, only 64.6 and 65.8% of correct STs predictions were obtained for the two considered mass ranges, demonstrating the current inadequacy of MALDI-TOF MS for discrimination of major *A. baumannii* STs. The difficulty to differentiate these STs based on MALDI-TOF MS analysis could possibly be associated with the relatedness of their allelic profiles. In fact, ST98 is a double locus variant of ST208 and ST218 in *gyrB* and *gpi* and ST208 a single locus variant of ST218 in the *gpi* allele. Despite the low ability to discriminate the four STs, *A. baumannii* ST103 isolates always presented the higher rate of correct ST predictions whether considering the comparison of the ST-mean mass profiles or the chemometric approach. It is of note that the allelic profile of ST103 isolates is the most dissimilar one, presenting only one common allele with ST98, the *gpi* one[3]. This fact could contribute to a more dissimilar ribosomal protein/peptide profile of ST103 isolates and its subsequent higher rate of correct identifications. The difficulty to differentiate these four STs based on their mass spectra suggests that these clones possess a very similar profile in what concerns to the molecules routinely observed in these MALDI experiments.

As a high throughput technique, MALDI-TOF MS competes with other spectroscopic techniques as Raman (Maquelin et al., 2006), and Fourier Transform Infrared Spectroscopy (FTIR) for bacterial typing at different taxonomic levels. It is of note, the suitability of FTIR to discriminate *A. baumannii* lineages, including the STs included in this study (Sousa et al., 2014b), which is also a sensitive, quick and low cost technique. Nevertheless, with the recognition of the interest in the microbiological diagnostic of MALDI-TOF MS, associated with the increasing availability of MALDI-TOF MS equipment in routine laboratories, there is a particular interest on methodology developments assisting bacterial epidemiology. In this way, further assays testing the ability of MALDI-TOF MS to discriminate *A. baumannii* lineages with different sample preparation conditions and matrix solutions should be conducted. It also should be noted that, despite our MALDI data had been compared with four distinct classification methods (two different MLST schemes, AFLP and SG), presenting different typing resolutions, it was not congruent with the grouping obtained with any of these approaches. In this way, it does not offer, in these experimental conditions, an advantage over other rapid methods such as DiversiLab rep-PCR-based typing, trilocus sequence-based typing, or single-locus-sequence-based typing of bla_{OXA-51}-like genes. However, we do not exclude the possibility that

[3]http://pubmlst.org/abaumannii/

other classification method could somehow fit MALDI-TOF MS data.

Conclusion

In this work we evaluate the ability of MALDI-TOF MS to discriminate *A. baumannii* clones. This mass spectroscopic technique, which revealed in previous studies a high discrimination power for species identification within the *A. calcoaceticus–A. baumannii* complex, demonstrates an insufficient result when used for discrimination at the clonal level. These findings suggest that the detected molecules, mainly ribosomal peptides and/or proteins, remain unchanged during clonal diversification in this species. Further studies, namely using different sample preparation conditions, are needed to provide further insights on the suitability of MALDI-TOF MS for typing *A. baumannii* at a subspecies level.

Acknowledgments

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00481/abstract

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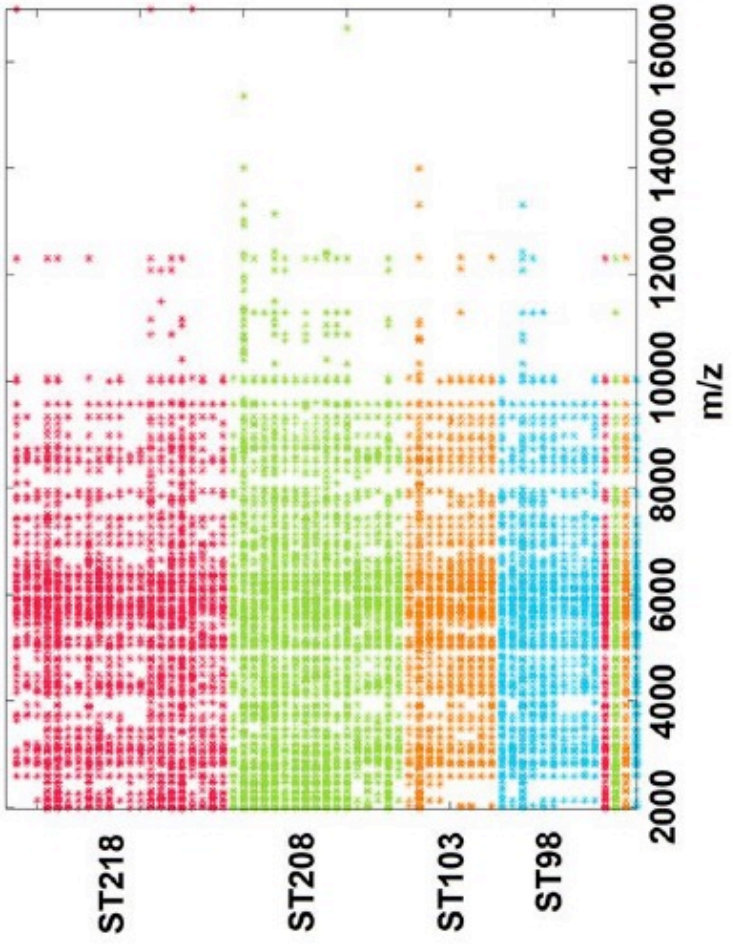
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



Peak positions (m/z) of the *Acinetobacter baumannii* isolates obtained with *peakfind* function from Matlab. The four lines at the bottom of the figure correspond to the mean-STs peak profiles (detailed description in MM section). Legend: * ST98, * ST103, * ST208 and * ST218.

The secret is on sugar: capsular type explains the discrimination of Acinetobacter baumannii clones by Fourier-transform Infrared (FT-IR) Spectroscopy and Multilocus Sequence Typing

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Manuscript Final Draft

1. Introduction

Acinetobacter baumannii is amongst the most troublesome Gram-negative bacterial pathogens worldwide, mainly due to the extended antibiotic resistance phenotypes and ability to rapidly disseminate and persist in healthcare settings (ECDC), European centre for disease prevention and control, 2017; Lee et al., 2017). In Portugal, we detected a recent endemicity of Sequence Type (ST) 218, which has been replacing the worldwide-disseminated ST208 since 2010 [3]. Surface polysaccharides are major virulence determinants that protect bacteria from phagocytosis and the bactericidal effect of serum, or host immune response, however, little is known regarding the capsular polysaccharides (KL) from *A. baumannii* major clones [4]. Moreover, in other bacteria it has already been established an association between capsular types and particular diseases or severity of infections [5]. Thus, it is of utmost importance to determine the role of *A. baumannii* capsule in host-pathogen interactions, as well as the genotypic and phenotypic features of *A. baumannii* KL, and how they affect the clonal dynamics among the major lineages associated with human infections.

This study aimed to characterize, by Fourier transform infrared (FT-IR) spectroscopy and whole genome sequencing (WGS), the capsular types of different *A. baumannii* isolates belonging to endemic/epidemic lineages, in order to correlate the capsular characteristics with the microevolution and predominance of specific STs.

2. Material and methods

Bacterial strains

One-hundred twenty-three previously characterized *A. baumannii* isolates from different clinical settings [two hospitals in the North (H, n=105) and Centre of Portugal (CB, n=11), a long-term care facility (LTCF, n=1) and a community clinical laboratory (CT, n=6)], belonging to different STs (ST98, n=2; ST103, n=2; ST208, n=22; ST218, n=93; sporadic STs, n=4) were studied [3,6]. With the exception of sporadic STs, they represent a snapshot of multidrug/extremely drug resistant (MDR and XDR, respectively) clones responsible for outbreaks or endemic situations in Portugal for a large period of time (2001-2015). Details about the bacterial isolates are summarized in Table 1.

FT-IR-ATR: spectra acquisition and processing

Isolates were grown on Mueller-Hinton agar (37ºC, 18h) and directly applied on the attenuated total reflectance (ATR) crystal. Spectra were acquired using a

Perkin Elmer Spectrum BX FTIR System spectrophotometer in the ATR mode (4000-600 cm^{-1}, 4 cm^{-1} resolution and 32 scan co-additions) and pre-processed as previously described [7]. Spectra were further analysed by an unsupervised (principal component analysis, PCA) chemometric method, considering the polysaccharides region (W_4, 900-1200 cm^{-1}) [8,9]. All chemometric analysis was performed using Matlab R2015a (MathWorks, Natick, MA) and PLS Toolbox version 3.5 for Matlab (Eigenvector Research, Manson, WA, USA).

Whole genome sequencing (WGS)

From the above collection, 16 *A. baumannii* isolates, representing different STs [ST98 (n=2), ST103 (n=2), ST208 (n=4), ST218 (n=4) and sporadic STs (n=4)], and time periods, were selected for WGS. DNA was extracted using the Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI), according with the manufacturer´s instructions. DNA concentration was estimated using the Qubit dsDNA HS Assay Kit and the Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, USA). Extracted DNA was then sequenced with a standard 2x125 paired-end runs protocol on Illumina HiSeq 2000 (Illumina, San Diego, CA, USA). The quality of the high-throughput sequence data was assessed by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw sequence reads were then *de novo* assembled using SPAdes v3.9.0 (http://bioinf.spbau.ru/spades), and the quality was assessed by QUAST (http://quast.bioinf.spbau.ru). RAST server (http://rast.nmpdr.org) was used for an automatic annotation that was further manually curated by Geneious R10 software (Biomatters Limited, Auckland, New Zealand) using BLASTn/BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Genetic characterization of KL locus

The full KL gene cluster, which encodes for capsule synthesis, assembly and export machinery, extending from the lipid II flippase (*mviN*) to L-lactate dehydrogenase (*lldD*) genes (approximately 30 kb), was extracted from whole genome data and annotated with Geneious R10. Easyfig (http://mjsull.github.io/Easyfig/) was used to compare the backbone of the different KL clusters.

3. Results and discussion

With FT-IR analysis, spectral diversity (92.2% in four principal components) revealed two well-defined clusters, I (n=23) and II (n=91), a stretched III (n=6), and

3 dispersed isolates (Figure 1). FT-IR-clustering, genetic characterization of the KL locus and MLST (using Oxford scheme, which includes *gpi* gene, contained in the KL locus and codifying for a glucose-6-phosphate isomerase) were, in general, congruent, unveiling putative associations between KL-types and particular STs over large periods of time.

Cluster I, encompassed all isolates from ST208 (n=22) sharing the same KL locus (KL_X). The same KL-type (100% identity) was also described among MDR strains AYP-A2 (CP024124.1) from Australia, CMC-CR-MDR-Ab4 (CP016295.1) and AF-673 (CP018256.1) from EUA, and a very similar one (99% identity) was described in South Korea (strain CBA7, CP020586.1), China (HRAB-85, CP018143.1) and Italy (strain ACICU, CP000863.1). This locus contains the operon *psaABCDEF* (Figure 2) that encode enzymes for the biosynthesis of pseudaminic acid, described for the first time in *Pseudomonas aeruginosa* (Pse5Ac7Ac). The only exception observed in this cluster, the isolate H366, belonged to a different clone (ST1558) and displayed a different KL gene cluster (with the operon *mnnABC* for the synthesis of mannose) (Figures 1 and 2). This exception makes us believe that, in addition to the KL-type, other factors [e.g. other sugars of the cell surface like the lipooligosaccharides (LOS)] could additionally influence the FTIR-based assignments.

Cluster II included ST218 isolates (n=91), with the same KL locus (KL_Y, 100% identity) of the MDR OXA-23-positive *A. baumannii* strains TCDC-AB0715 from Taiwan and BJAB0868 from China (both belonging to ST2 or Global clone II) [10,11]. Interestingly, this locus is associated with genes (glycosyltransferases and the operon *lgaABCDEF*) involved in the biosynthesis and processing of legionaminic acid (Leg5Ac7R), the precursor for the uncommon sugar α-8-epi-legionaminic acid (Figure 2). This sugar, like the pseudaminic acid from ST208, belongs to the superfamily of nonulosonic acids (complex nine carbon α-keto monosaccharides) that have structural similarities with the eukaryotic sialic acid [12]. However, these two acidic sugars have different stereochemistry, with legionaminic acid better resembling the structure of sialic acid from mammalian host cell´s surface glycoproteins, enabling the bacterial pathogens to mimic host cell surface, and thus escaping from host immune surveillance to facilitate their colonization and invasion [12]. Although this relation needs further studies, this sugar may constitute an advantage for ST218 isolates, which could be one of the reasons for its success in relation to ST208 [3]. It is of note an isolate belonging to ST218 (H482, Figure 1) that appears apart from the cluster. However, the only capsular genetic difference found between this isolate and the remaining ST218 isolates was an alteration in the tyrosine protein kinase, Wzc (insertion of the TCT codon on *wzc* gene sequence,

which leads to the synthesis of an extra lysine amino acid at the position 499). Wzc is a protein and, hence, is not included in the region used for FTIR analysis. However, its essential function in capsule export makes us believe that some modification in this process may have occurred, pointing out the need of further studies.

Cluster III (n=6) encompassed isolates from different STs: Ac55 and H25 from ST98, H735 and H736 from ST218, and Ac23 and Ac246 from ST103 (Figure 1). Interestingly, the analysis of the KL clusters revealed that all of these isolates shared the same capsular locus (KL_Z, 100% identity), which possessed the *fnlABC* operon, responsible for the biosynthesis of N-acetyl-L-fucosamine.

Dispersed isolates (n=3) were unrelated, belonged to sporadic STs (ST234, ST552 and ST1557) and harboured different KL-types (Figures 1 and 2). Despite the capsular locus of two of these isolates contain the operons *psaABCDEF* (316.1) and *rmlBADC* (H745) for the synthesis of pseudaminic acid and rhamnose, respectively, they do not present the polymerase-encoding gene (*wzy*), crucial for the sugar-linked repeat units polymerization. This observation corroborated a previous study performed by Dalong Hu and colleagues [13], who did not find the pseudaminic sugar in a capsular structure similar to that observed for 316.1 isolate, suggesting that these sugars are not incorporated into the capsular structure. Moreover, none of the KL-types harboured by sporadic isolates appear to be widespread or related with an epidemic ST.

Taken together, the results obtained evidenced a good correlation between FT-IR-based assignments and the genetic assessment of the KL locus. An apparent low potential for KL diversification within each of *A. baumannii* clones studied was observed (being a clone usually associated with a specific capsule), highlighting the role of clonal selection in the actual epidemiological scenario of *A. baumannii,* at least in the clinical setting. However, when subtle changes on bacterial surface components or a capsular switch occurs, FT-IR spectroscopy proved to have a good discriminatory potential to identify them, which can be useful to detect alterations/switch, as a result of host-pathogen adaptation or recombination events, in the local population of *A. baumanni* involved in infections. Furthermore, although the presence of legionaminic and pseudaminic acid in *A. baumannii* capsules have been punctually recognized in MDR isolates [14,15], we firstly clarify the abundance of these sugars among KL-types of a representative collection of *A. baumannii* MDR/XDR strains.

4. Conclusions

This work demonstrated, for the first time, the potential of FT-IR coupled with chemometric analysis to differentiate *A. baumannii* capsular types (at least in lineages with clinical significance), confirming the suitability of this method for a quick and low-cost bacterial characterization. Although the results obtained have shown that the capsular gene cluster do not unambiguously correlate with any particular ST (e.g. ST218 can be equipped with at least 2 different types of gene clusters, although one clear predominant), the high prevalence of capsule derivatives enriched in acidic sugars and apparently more adapted to the host interaction, among the international XDR *A. baumannii* lineages ST208 and ST218, in different geographic regions and time periods is of relevance, and needs to be further explored.

Transparency declarations: None to declare.

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Table 1. Epidemiological data and genotypic characterization of *A. baumannii* clinical isolates analysed in this study.

	ST (Oxford scheme) (no.)	Representative Strains	Year of isolation/Origin[a]	Acquired CHDL	FTIR Cluster	Capsular sugar composition
Epidemic STs	**98 (n=2)**	**Ac55**	2002/H	OXA-40	C_{III}	Fucosamine
		H25	2006/H	OXA-40	C_{III}	Fucosamine
	103 (n=2)	**Ac23**	2001/H	OXA-58	C_{III}	Fucosamine
		Ac246	2004/H	OXA-58	C_{III}	Fucosamine
	208 (n=22)	**H466**	2011/H	OXA-23	C_{I}	Pseudaminic acid
		H580	2012/H	OXA-23	C_{I}	Pseudaminic acid
		H603	2013/H	OXA-23	C_{I}	Pseudaminic acid
		H678	2013/H	OXA-23	C_{I}	Pseudaminic acid
	218 (n=93)	**H202**	2010/H	OXA-23	C_{II}	Legionaminic acid
		H482	2012/H	OXA-23	Dispersed	Legionaminic acid
		H631	2013/H	OXA-23	C_{II}	Legionaminic acid
		H735	2014/H	OXA-23	C_{III}	Fucosamine
Sporadic STs	**234 (n=1)**	**H745**	2014/H	-	Dispersed	Rhamnose
	552 (n=1)	**H637**	2013/H	-	Dispersed	-
	1557 (n=1)	**316.1**	2015/LTCF	-	Dispersed	Pseudaminic acid
	1558 (n=1)	**H366**	2011/H	-	C_{I}	Mannose

[a] **H**, Hospital located at North of Portugal; **LTCF**, Long-term care facility.

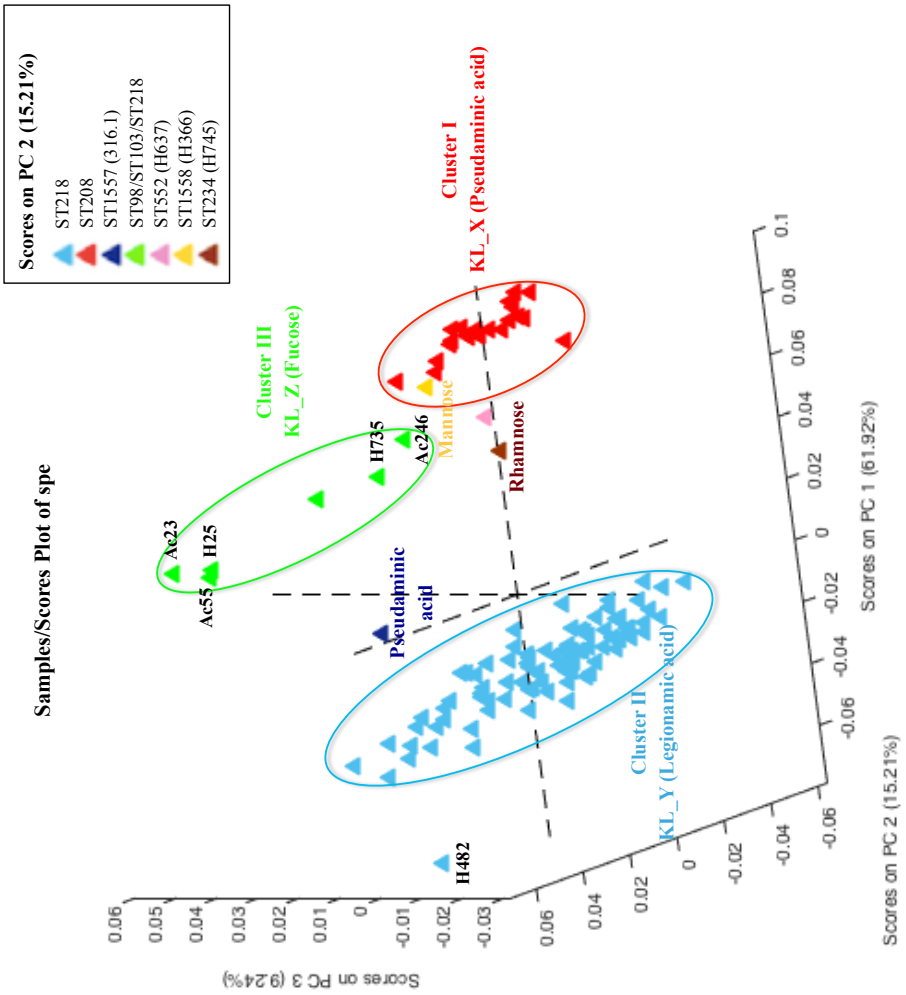
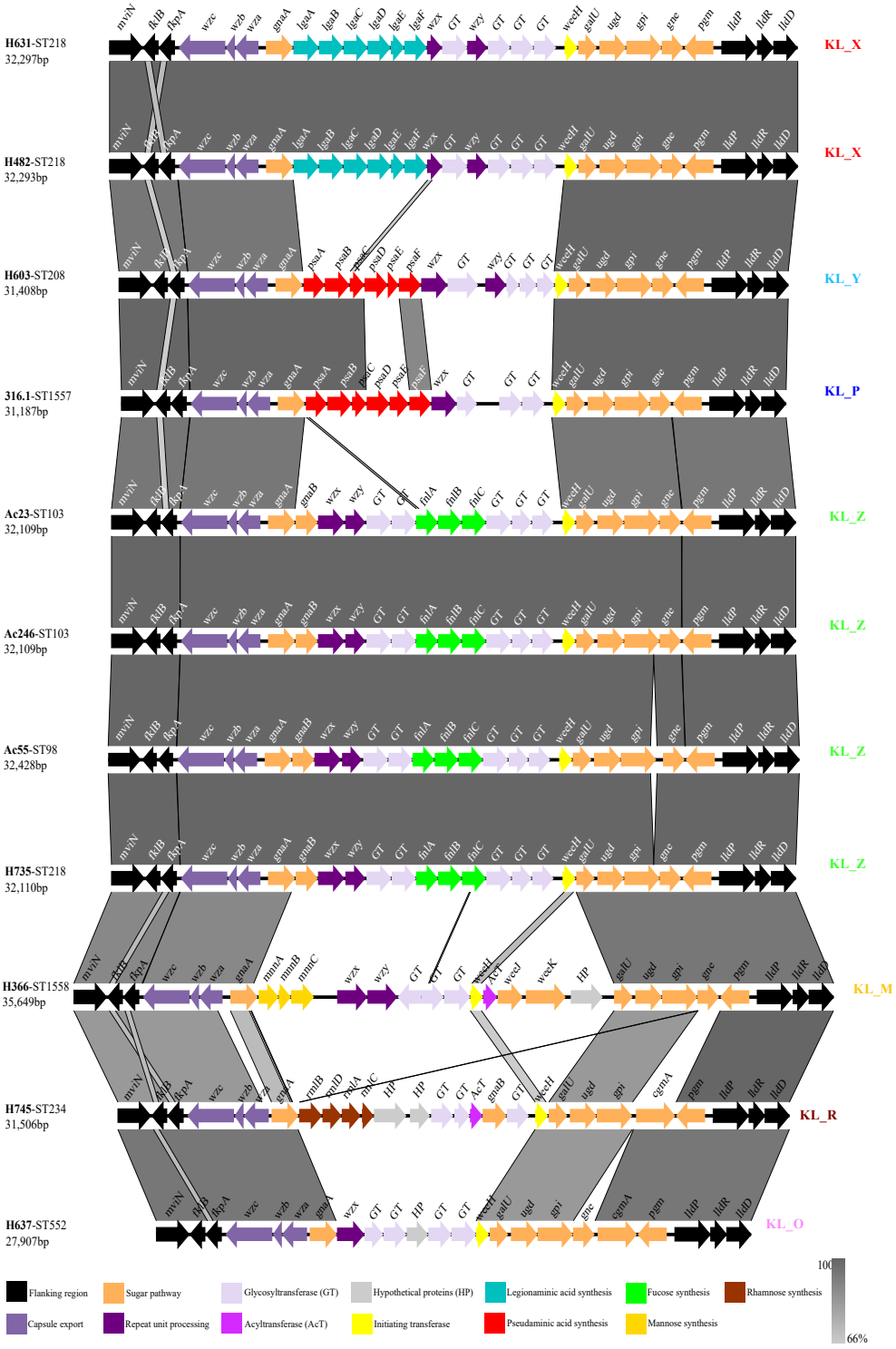
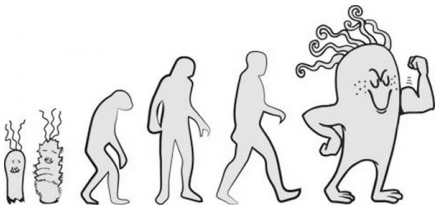


Figure 1. PCA of the different *A. baumannii* clones established by analysis of FTIR-ATR spectra.

Figure 2. Comparison of the KL gene clusters from *A. baumannii* representative strains. The STs for the respective strains and cluster length (on the left side), and the predicted KL-types (on the right side) were also shown. Homologous regions are connected by areas of different shades of grey, reflecting the degree of nucleotide identity (from 66% to 100%)





Chapter 4

Conclusions

"Nobody said it was easy
No one ever said it would be this hard."
Coldplay, "The Scientist"

The **general conclusions** that can be extracted from the achievement of the specific aims proposed are summarized as follows:

Carbapenem-resistant *Acinetobacter baumannii* clonal dynamics in Portuguese clinical settings explained by a highly successful interplay between antimicrobial resistance and virulence.

1. For more than two decades, high rates (>45%) of carbapenem-resistant *A. baumannii* have been reported in Portuguese clinical settings, associated in each time period with a particular ST. **ST218**, which gradually replaced the previously dominant lineage **ST208**, is now the main lineage contributing for **carbapenem-resistance** among the Portuguese clinical settings analyzed. Based on a detailed genomic analysis of these clones (obtained by WGS), a better understanding of clonal replacement was provided. It was observed that **higher levels of aminoglycoside resistance**, mainly due to the presence of 16S ribosomal RNA methyltransferase ArmA enzyme; an **enlarged virulence potential**, in particular due to the heme oxygenase, essential for uptake and utilization of iron, and a **capsular type** mimicking the host cell surfaces and enabling the bacteria escape from the immune system, are the main advantages displayed by ST218 when compared with ST208.

2. Despite the lineage substitution, chromosome located ***bla*$_{\text{OXA-23}}$** is still the most prevalent carbapenem resistance determinant associated with *A. baumannii* during the analyzed period (2010-2015). Curiously, no antibiotic resistance associated-plasmids were found among the *A. baumannii* collection, except one isolate found in a LTCF and belonging to a sporadic clone (ST1557), which displayed *bla*$_{\text{OXA-24/40}}$ in a plasmid identical to pAc92, previously associated with the ST98 lineage.

Contribution of *non-baumannii Acinetobacter* species as reservoirs of resistance genes in clinical and non-clinical settings.

3. A clear potential of **non-*baumannii Acinetobacter*** species to acquire and act as a source of carbapenem-resistance determinants, with the ability to integrate different genetic backgrounds and potential for further dissemination, was observed. Two new CHDL-carrying plasmids (***bla*$_{\text{OXA-23}}$**-carrying pLS488 and the ***bla*$_{\text{OXA-58}}$**-carrying pLS535) were identified among ***A. pittii***, a species increasingly detected causing infection in Portuguese hospitals. The pLS488 (51 Kb) displayed a **new replicase** (*rep*) gene that belonged to the superfamily RdgC, and was the first **conjugative plasmid** associated

with a carbapenemase gene in this species, which was successfully transferred to *A. baumannii*. The pLS535 (12 Kb) belonged to the GR7 incompatibility group (Rep3 superfamily) and, although lacked the conjugation machinery was transferable by electroporation to *A. baumannii*. In addition, the ***bla*$_{\text{IMP-5}}$** gene, previously detected among *A. baumannii* and *Pseudomonas aeruginosa* clinical isolates, was identified for the first time in two ***A. bereziniae*** isolates, one from hospital equipment and other causing infection. In both isolates, *bla*$_{\text{IMP-5}}$ gene was associated with large transferable plasmids, embedded in the same genetic element previously reported (In76), which highlights the possibility of interspecies transfer and may contribute for further dissemination of important carbapenemases among ***Acinetobacter***.

4. A high diversity of *Acinetobacter* species, scattered in different niches of Benguela (Angola) was observed, enlarging their recognized niches. The occurrence of a ***bla*$_{\text{OXA-58}}$-producing *A. johnsonii*** and ***bla*$_{\text{OXA-23}}$-producing *A. towneri*-like species** (a potential new species) from a non-related-hospital source was here firstly reported, suggesting the involvement of unknown stressors for the emergence of these relevant resistance genes. Worrisome, was identified in this region, a possible water-human transmission cycle for *Acinetobacter* spp., including CHDL producers.

Developments on *Acinetobacter* species identification and typing.

5. It is demonstrated, for the first time, the potential of Fourier Transform infrared (FTIR) spectroscopy, coupled with chemometric analysis, for the **identification of closely related species** belonging to the ACB complex, and also for the discrimination of clinically relevant *A. baumannii* lineages (ST98, ST103, ST208 and ST218, Oxford scheme). Moreover, it was possible to correlate the differences observed in FTIR-assignments with the capsular gene clusters harboured by MDR/XDR *A. baumannii* clones. Our data points to a **high resolution of FTIR-ATR coupled with chemometric analysis for capsular types identification**.

6. We demonstrate the suitability of MALDI-TOF MS for **species identification** within the **ACB complex.** Nevertheless, a **limited discriminative potential for MDR *A. baumannii* clones was verified**, which might be explained by the high degree of conservation of ribosomal proteins.

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